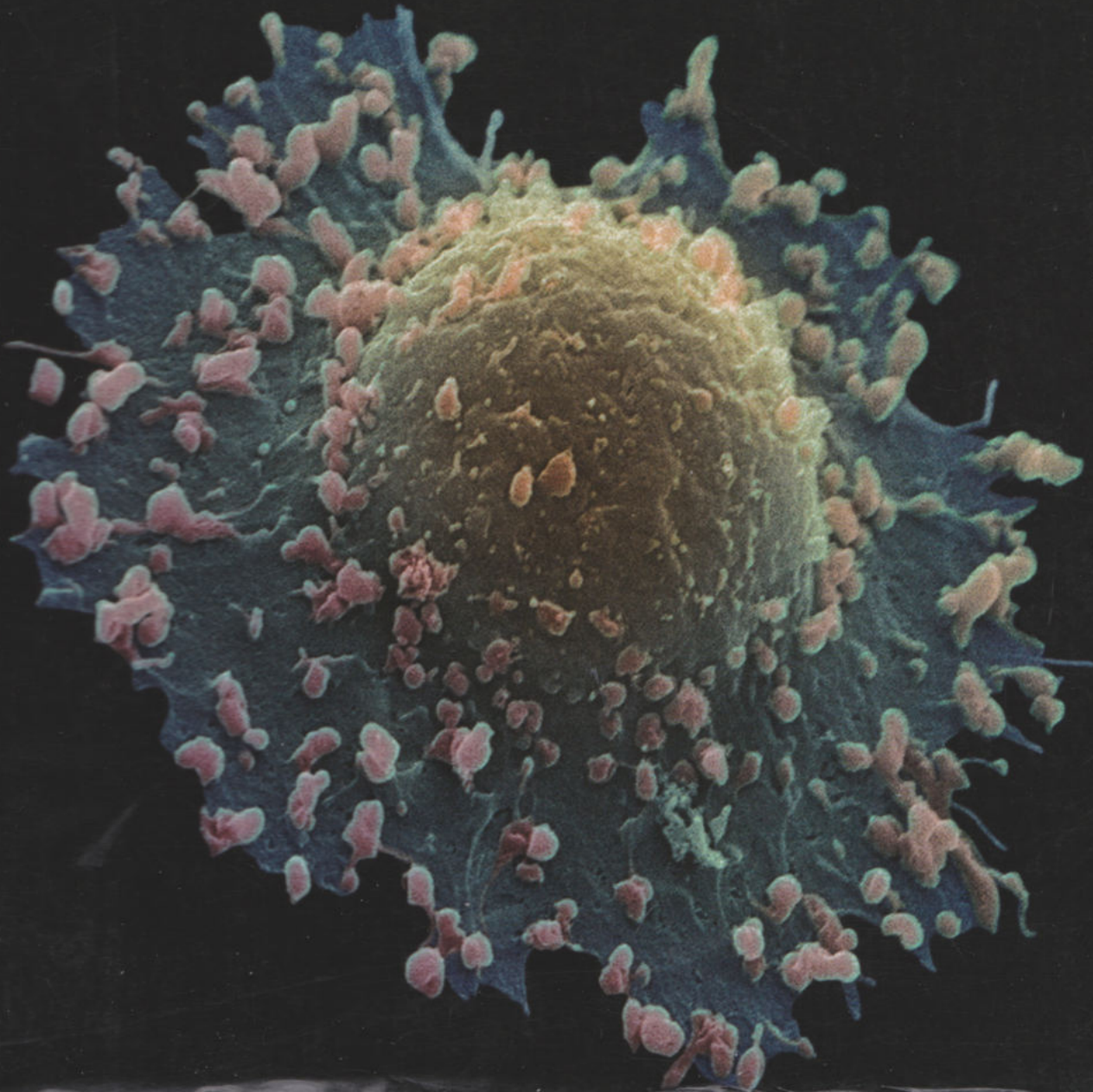




Book 4

The Interactive Cell

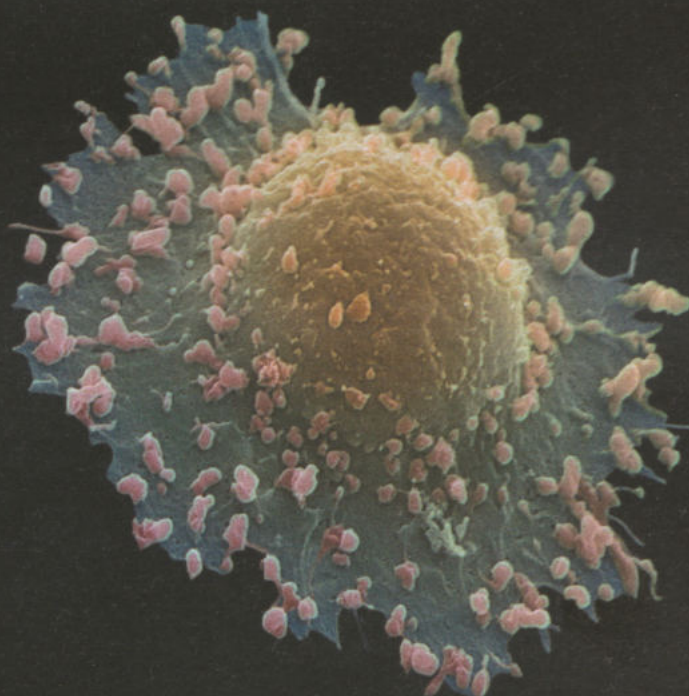




The Open University

The Interactive Cell

prepared for the Course Team by
Sally Cowley, David Male, Ignacio Romero and Jill Saffrey



The S377 Course Team

Course Team Chair

David Male

Course Manager

Viki Burnage

Colin Walker

Course Team Assistants

Rebecca Efthimiou

Lara Knight

Course Team authors

Mark Hirst

Jane Loughlin

David Male

Radmila Mileusnic

Sotiris Missailidis

Ignacio Romero

Jill Saffrey

Robert Saunders

Consultant author

Sally Cowley

Editors

Ian Nuttall

Gilly Riley

Dick Sharp

Margaret Swithenby

Academic Reader

Christine Gorman

External Course

Assessor

Iain Campbell

OU Graphic Design

Roger Courthold

Sara Hack

Jenny Nockles

Video production

Wilf Eynon

Michael Francis

CD-ROM production

Greg Black

Eleanor Crabb

Hilary MacQueen

Rights Executive

Chris Brady

Picture Research

Lydia Eaton

Indexer

Jane Henley

Course website

Patrina Law

Louise Olney

The Open University, Walton Hall, Milton Keynes, MK7 6AA, United Kingdom

First published 2004. Second edition 2008

Copyright © 2004, 2008 The Open University

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, transmitted or utilized in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without written permission from the publisher or a licence from the Copyright Licensing Agency Ltd. Details of such licences (for reprographic reproduction) may be obtained from the Copyright Licensing Agency Ltd of 90 Tottenham Court Road, London W1T 4LP.

Edited, designed and typeset by The Open University.

Printed and bound in the United Kingdom by Halstan Printing Group, Amersham.

ISBN 978 07492 26763

This publication forms part of an Open University course S377 *Molecular and Cell Biology*. Details of this and other Open University courses can be obtained from the Course Information and Advice Centre, PO Box 724, The Open University, Milton Keynes MK7 6ZS, United Kingdom: tel. +44 (0)1908 653231, e-mail general-enquiries@open.ac.uk

Alternatively, you may visit the Open University website at <http://www.open.ac.uk> where you can learn more about the wide range of courses and packs offered at all levels by The Open University.

To purchase a selection of Open University course materials visit the webshop at www.ouw.co.uk, or contact Open University Worldwide, Michael Young Building, Walton Hall, Milton Keynes MK7 6AA, United Kingdom for a brochure. tel. +44 (0)1908 858785; fax +44 (0)1908 858787; email ouwenq@open.ac.uk

S377 Book 4 i2.1

CONTENTS

15	CELLULAR INTERACTIONS	1
	David Male	
15.1	Introduction	1
15.2	The relationship of cells to organisms	2
	Learning outcomes for Chapter 15	6
	Reference	6
16	CELL MIGRATION AND ADHESION	7
	David Male	
16.1	Introduction	7
16.2	Cell adhesion molecules (CAMs)	13
16.3	Control of cell adhesion in cell migration	27
16.4	Cell movement	32
16.5	Chemotactic molecules and their receptors	37
16.6	Control of cell adhesion and chemotaxis	42
16.7	Disintegrins and metalloproteases	46
	Learning outcomes for Chapter 16	47
	Questions for Chapter 16	48
	Reference	48
	Further sources	48
17	DIFFERENTIATION OF ANIMAL CELLS	49
	Jill Saffrey	
17.1	Introduction: what is differentiation?	49
17.2	Common mechanisms and molecules underlie differentiation	51
17.3	Differentiation of the nervous system during development	71
17.4	Differentiation and plasticity in mature tissues	89
	Learning outcomes for Chapter 17	97
	Questions for Chapter 17	97
	References	98
	Further sources	98

18	CELL AGEING AND SENESCENCE	99
	Jill Saffrey	
18.1	Introduction	99
18.2	Free radicals and ageing	101
18.3	Ageing of mitotically active cells: replicative senescence	108
18.4	Ageing of post-mitotic cells	119
18.5	Genomic instability and ageing: lessons from progeroid syndromes	123
18.6	The insulin signalling pathway; ageing of cells and organisms	124
	Learning outcomes for Chapter 18	125
	Questions for Chapter 18	126
	References	126
	Further sources	126
19	TUMORIGENESIS	127
	Sally Cowley and Ignacio Romero	
19.1	Introduction	127
19.2	Genetic instability	136
19.3	Self-sufficiency in growth signals	142
19.4	Insensitivity to growth-inhibitory signals	157
19.5	Evasion of apoptosis	162
19.6	Limitless replicative potential	164
19.7	Sustained angiogenesis	168
19.8	Tissue invasion and metastasis	170
19.9	From tumour cells to cancer	173
	Learning outcomes for Chapter 19	177
	Questions for Chapter 19	178
	References	178
	Further sources	179
	EPILOGUE	180
	ANSWERS TO QUESTIONS	181
	ACKNOWLEDGEMENTS	184
	INDEX	186

15

CELLULAR INTERACTIONS

In Books 2 and 3, we looked at the functions of cells and how they relate to subcellular organization. In the last two chapters – concerned with signal transduction and cell death – we started to introduce some cellular functions that are particularly important in multicellular organisms, which have differentiated tissues and high levels of cellular interaction and cooperation. These themes are developed further in the present book, where we discuss additional cellular functions and relate these to some of the cellular interactions seen in complex organisms. The sheer diversity of living creatures means that the themes chosen are highly selective, but they illustrate important general features. Cell migration and differentiation (Chapters 16 and 17) are properties of all multicellular animals, while cell ageing and tumorigenesis (Chapters 18 and 19) are two topics that have additional important implications for biomedical science.

One of S377 learning outcomes relates to the skill of critically reviewing and assessing the primary scientific literature. Based on the experience you have gained through your studies of the earlier course materials, you will undertake reading activities on a paper or papers related to topics covered in Chapters 16, 17 or 18 of this book. These *Reading scientific literature* activities can be found in the Study Skills file, and you should refer to the S377 Study Calendar for the suggested study dates.

15.1 Introduction

For the remainder of this course, we are going to look at ways in which individual cells relate to the whole organism, and to other cell types within it. The intracellular signalling systems described in Chapter 13 are essential for a cell to recognize and act appropriately in response to an extracellular signal. Here, though, we will be considering cells not only as single living entities but also as members of complex interactive networks with other cells of the same or a different type and in the same or in another organ or system. In this context, it is important for you to think in terms of the relationship between individual cells and between cells and the organism they belong to, a relationship that affects: cell numbers and cell division; cell positioning and contacts with other cells and with the extracellular matrix; and cell functions.

- ☐ In what two principal ways can a cell interact with other cells or signal to them?
- ☒ (1) By responding to or secreting soluble factors, such as cytokines, hormones, neurotransmitters and small signalling molecules, which act on receptors mostly at the cell surface. (2) By direct cell–cell contact mediated by proteins bound to the plasma membrane of each cell. (See Section 13.1.1.)

In this book, we go beyond the understanding of individual cellular processes and consider the functioning of cells in a social context. In order to do this, we will explore a number of important integrative and specialized cellular activities, which are briefly introduced in this chapter.

15.2 The relationship of cells to organisms

15.2.1 Cell numbers and cell division

In multicellular organisms, particularly animals, there is a close relationship between the number of cells of any particular type and the organism as a whole. For example, if part of the liver in a mammal is removed by surgery, cells in the remainder of the organ will divide, so that it regrows to its original size and in a particular shape. Similarly, during development, many different organs with a defined set of cell types will form in a precise way and in an orchestrated fashion, so that gross variations in organ size and shape between individual animals are kept to a minimum. The ability of organs to grow into a defined shape and size demonstrates a clear link between the size of the organism, its metabolic requirements and the number of cells produced to fulfil those requirements. What extracellular signals determine cell numbers in an organ or system?

The relationship of cell number to the metabolic requirements of an organ or system was first demonstrated for erythrocytes (red blood cells). A primary determinant of erythrocyte number is the availability of oxygen in the tissues. Hypoxia, i.e. a fall in available tissue oxygen, results in the production of additional erythrocytes to increase the oxygen-carrying capacity of the blood – an effect seen in climbers who spend extended periods at high altitude where atmospheric oxygen levels are low (Figure 15.1). The control of erythrocyte numbers in response to hypoxia is mediated by erythropoietin, a hormone produced by cells in the kidney. Hypoxia or blood loss produce a rise in the blood levels of erythropoietin within

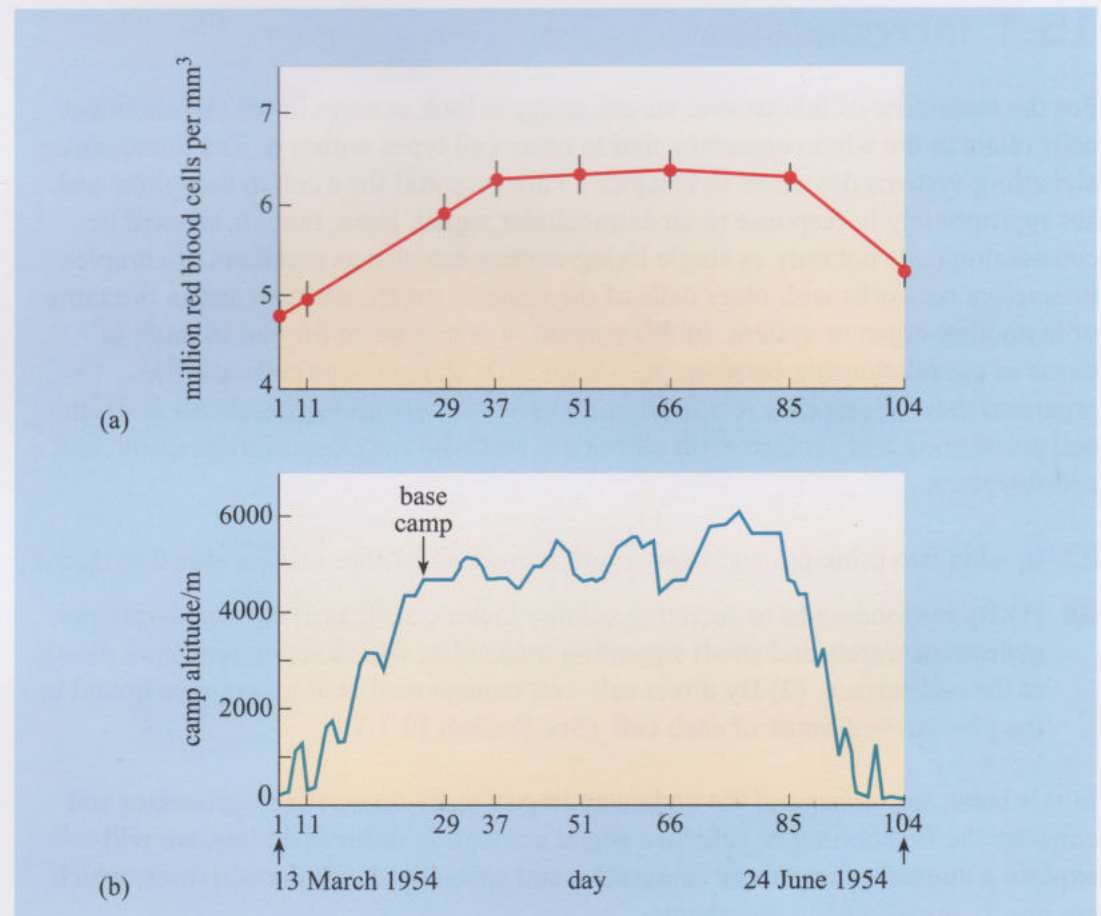


Figure 15.1

(a) The mean red blood cell count compared with (b) the mean altitude in a group of ten expedition members who attempted to reach the summit of Makalu (Tibet) in 1953. The group spent 62 days continuously above 15 000 ft (4000 m), and erythrocyte numbers increased over the first 5 weeks, in response to hypoxia. (Data from Meyer *et al.*, 1956)

hours, which in turn induces the differentiation of erythrocyte progenitor cells in the bone marrow into mature erythrocytes. An increase in erythrocyte production and release from the bone marrow into the bloodstream then follows. When the oxygen-carrying capacity of the blood is restored, erythropoietin levels fall.

The identification of erythropoietin and its effect on erythrocytes led to searches for cytokines and hormones that control the division of other cell types. (Cytokines and hormones that have cell proliferative effects are collectively known as growth factors.) For example, several cytokines that modulate leukocyte development have been identified (Figure 15.2). There are more than 200 different types of differentiated cells in a mammal, so it is thought that there must be at least the same number of growth factors (or at least of combinations of a smaller number of growth factors), to determine the numbers of cells in each tissue or organ. Although we know something of this subject, for most tissues the precise way in which cell numbers are matched to the requirements of the organism is not known.

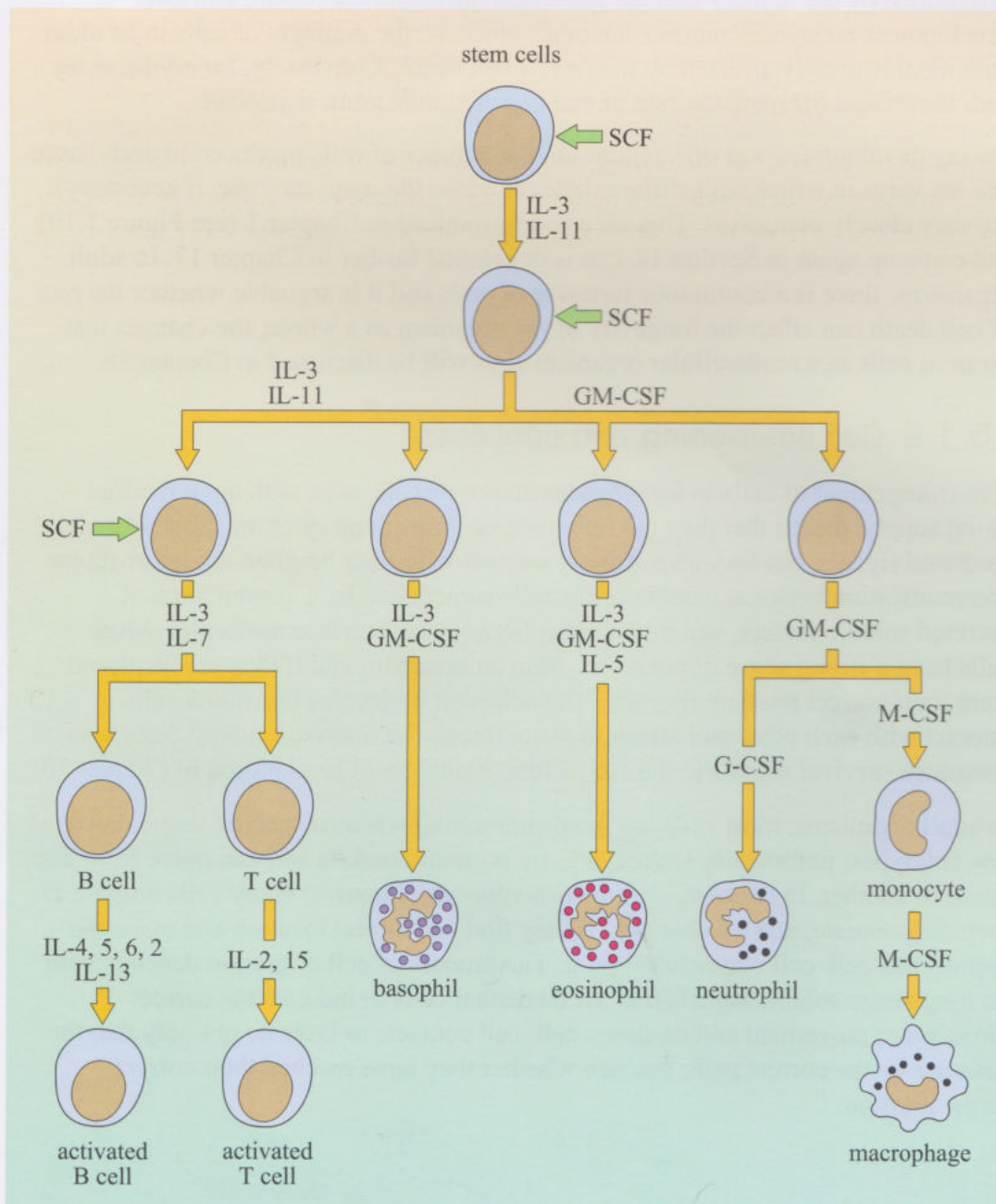


Figure 15.2

Cytokines that control the division and differentiation of different lineages of leukocyte are illustrated. The target cells include stem cells that develop into B and T lymphocytes, basophils, eosinophils, neutrophils and macrophages. Note that stem cell factor (SCF) principally promotes cell division (green arrows), while other cytokines also promote differentiation. IL-2, IL-3, etc. indicate interleukins; GM-CSF = granulocyte macrophage colony stimulating factor; G-CSF = granulocyte colony stimulating factor; M-CSF = macrophage colony stimulating factor.

- ☐ What would the consequences be for a cell that is not subject to the normal controls on cell division?
- ☒ The cell will divide inappropriately, which increases the cell mass within the tissue (a process called hyperplasia). If this process continues, a solid tumour may develop within the tissue.

You can see that as a cell loses successive negative controls on its division and/or receives inappropriate signals to divide, it becomes increasingly malignant. This subject is explored further in Chapter 19.

Cell division is only one half of the equation. The number of cells in an organ or a system is also determined by the rate of cell death, which varies depending on the cell type. For example, erythrocytes have a lifespan of about 120 days, neutrophils (a type of leukocyte) around 48 hours, lymphocytes days to years, and neurons may last a lifetime. For cells such as neurons, the starting cell population is initially determined by the number that are generated during development, and after development no significant division takes place, so the numbers of cells in an older individual is greatly affected by the rate of cell death. Conversely, for erythrocytes with their fixed lifespan, the rate of cell production is more important.

During development and differentiation, the number of cells produced in each tissue and the ways in which cells differentiate (and also the ways they die, if necessary) are very closely controlled. This idea was introduced in Chapter 1 (see Figure 1.10) and came up again in Section 14.1; it is developed further in Chapter 17. In adult organisms, there is a continuous turnover of cells and it is arguable whether the rate of cell death can affect the longevity of the organism as a whole; the changes that occur in cells as a multicellular organism ages will be discussed in Chapter 18.

15.1.2 Cell positioning and contacts

The arrangement of cells to form tissues and/or organs, each with an individual blood supply, means that they not only receive long-range cytokine- and hormone-mediated signals, but they also directly interact with their neighbours. Intercellular communication between neighbouring cells is mediated by a combination of secreted soluble factors, and interactions between cell surface molecules. Most cells have a strong sense of position within an organism and if they are displaced from their correct position they die. The adhesion molecules that allow cells to interact with each other and attach to the extracellular matrix, in many cases also transduce survival signals to the cell, a function that will be explored in Chapter 16.

In adult organisms, most cells are fixed (immobile) within a specific tissue, but a few cell types, particularly leukocytes, are normally mobile and can move from one tissue to another. In contrast, during embryonic development many cells migrate as they differentiate, and survive only if they find the correct location and make the appropriate cell–cell contacts within it. The process of cell migration depends both on long-range soluble signals that act on distant cells to indicate the correct direction of movement and on direct cell–cell contacts to indicate not only that the cells are on the correct path, but also whether they have reached their correct target location.

The term cytokine is generally used for short-range polypeptide signalling molecules that act locally. However, many cytokines are secreted by cells that are either circulating in the blood (immune cells) or are part of the structure of blood vessels (endothelial cells), and are therefore transported through the blood to act on distant cellular targets.

- ☐ What would the consequences be for tumour cells that have lost their positional controls?
- ☒ They may become mobile, invade other tissues and survive in another tissue – in other words, they may progress from a solid tumour into an invasive cancer (Chapter 19).

15.1.3 Cell functions

Most intercellular communication that takes place in a developed multicellular animal affects, in one way or another, the functions of the responding cells. As with cell division and movement, many other cell functions can be modulated by soluble factors, direct cell–cell contacts or both. The ways in which different cell types and tissues interact with each other in an organism is the subject of physiology, which is beyond the scope of this course. However, the overall response of an organism is based on the responses of the individual cells, produced primarily by changes in the expression or function of individual proteins.

- ☐ How can extracellular signalling molecules, such as cytokines, change the expression or function of a cellular protein, i.e. what is the chain of events within the responding cell?
- ☒ The signalling molecule binds to a receptor, which activates intracellular signalling proteins and second messenger systems (Chapter 13). One of the possible cellular outcomes may be the activation and/or assembly of transcription factors, which cause RNA transcription (Chapter 10) and new protein synthesis (Chapter 11). Alternatively, extracellular signals may induce cytoskeletal changes (Chapter 12) or alter cellular metabolism.

Signal transduction pathways and cellular effects are closely related to the speed of the response required, as illustrated by the three examples given in Table 15.1. Notice that the signals can affect protein function directly, protein localization/availability, or protein expression. You might compare this with Table 7.1 which also relates changes in the activity/location/expression of proteins to the speed of response.

Table 15.1 Examples of intercellular communication.

Speed of response	Signal molecule	Target	Cellular response	Physiological effect
milliseconds	acetylcholine	skeletal muscle	opens ligand-gated Na ⁺ channels	skeletal muscle contraction controlled by nerve endings (Chapter 13)
minutes	insulin	liver hepatocyte	mobilizes glucose transporters from intracellular stores	glucose uptake and fall in blood glucose levels (Chapter 13)
hours	interleukin-4	B lymphocyte	activates protein kinase cascade and transcription factors	cell division as part of an immune response (Chapter 12)

The final example in Table 15.1 is the stimulation of B lymphocyte division by interleukin-4, a cytokine released by T helper cells. However, when we introduced this interaction in Section 12.6.5, we noted that the B cells also require a direct cell–cell interaction with another cell type – a T cell.

- ☐ What combination of molecules on the B cell surface is recognized by the T cell, and what is the interaction between the two cell types called?
- ☒ T cells recognize MHC class II molecules with antigen peptides bound to them, a complex expressed on the cell surface of B cells. The interaction between the two cell types is called antigen presentation.

This example is illustrative of many cellular interactions between adjoining cells, which involve both direct interactions and communication mediated by soluble signalling molecules.

Summary of Chapter 15

- 1 The numbers of differentiated cells of each type is determined by the requirements of the organism, and is affected by both the rate of cell production and the rate of cell death.
- 2 Cell division occurs during development, and for many cells replacement (as a result of division of precursor or stem cells) occurs during adult life when necessary. Growth factors such as erythropoietin can signal division and/or differentiation and therefore affect cell numbers.
- 3 Cell positioning is signalled by direct cell–cell contacts, and via soluble factors. During development, differentiating cells find their correct position in the embryo and divide appropriately. In the adult, most cell types are fixed and only divide if extracellular signals are present.
- 4 Failure to respond appropriately to signals controlling cell division and cell position can lead to tumour development and progression.
- 5 Changes in cell function involve changes in protein activity, localization and/or expression. The speed of a physiological response to an extracellular signalling molecule is related to the activated network of signal transduction pathways leading to effects specific for an individual cell type.

Learning outcomes for Chapter 15

When you have studied this chapter you should be able to state the two broad categories of cellular interaction, give examples of each type, and say how these interactions can affect cell numbers, position or function of the responding cell.

Reference

Meyer, L. B., Pace, N. and Vaughan, B. E. (1956) Erythrolysis on return of altitude-acclimatized individuals to sea level, *Journal of Applied Physiology*, **9** (2), pp. 141–144.

16 CELL MIGRATION AND ADHESION

16.1 Introduction

In this chapter we shall be looking at cell adhesion and cell migration, two processes that are closely interrelated, and very important for the development and function of multicellular organisms. Cell contacts with the extracellular matrix and with other cells were first introduced in Chapter 6. As an example, Figure 6.35 showed how epithelial cells from the gut can attach to the *basal lamina* and may bind to other cells via intercellular junctions.

- ☐ Name the different types of intercellular junction that occur in different cell types, and state some of their functional characteristics.
- ☒ Tight junctions act as barriers to the lateral diffusion of molecules in membranes and, for example, separate the apical and basolateral domains of the plasma membrane in gut epithelial cells. Anchoring junctions allow cells to attach to the extracellular matrix (focal adhesions and hemidesmosomes) or to each other (desmosomes and adherens junctions). For example, they are found in sheets of epithelial cells. Gap junctions allow the passage of small molecules and ions between cells and so may be involved in direct intercellular signalling (see Section 6.6.2).
- ☐ What types of transmembrane molecule are associated with anchoring junctions?
- ☒ Cadherins and integrins (Section 6.6.2).

Specialized cell junctions can be classified into three main groups: (a) tight or occluding junctions, which seal cells together; (b) anchoring junctions, which mechanically attach cells; and (c) communicating junctions, which allow rapid transfer of chemical or electrical signals (this type includes gap junctions and neuronal synapses). The type of adhesion mediated by cell junctions is generally referred to as 'junctional cell adhesion'. However, specialized mature junctions are not the only types of cell contacts. Other cell contacts may involve transient interactions with other cells or with the extracellular matrix as cells move in one direction, or they may involve initial points of contact before establishing mature cell junctions, both processes that we shall include under the general term of 'non-junctional cell adhesion'.

At first sight, it might appear that cell migration is exactly the opposite of cell adhesion, since a migrating cell loses its position and extensive contacts with its neighbours. However, most migrating cells in multicellular animals can only move by interacting with a solid support and have to retain some contact with other cells or the basal lamina or the substratum (the solid surface over which a cell moves). An exception to this rule is cells that move by swimming. Cell movement is a basic function of many cell types, and is seen in the simplest single-celled organisms as they swim towards nutrients, respond to light, or avoid toxic chemicals in their environment. Single-celled organisms may move by means of cilia or flagellae, which are specialized structures formed from bundles of microtubules; these

The layer of extracellular matrix that lies under sheets of cells (such as epithelial cells) is called the 'basal lamina'. It is sometimes called 'basal membrane' but, in fact, basal membranes are now more often referred to as *basal laminae*, to emphasize the fact that they are not phospholipid bilayers, but layers of extracellular matrix.

structures are clearly related to the eukaryotic cytoskeleton. However, in multicellular animals, the requirements for cells to move by swimming have almost completely disappeared, and in higher animals this function is effectively confined to sperm cells. In contrast, cell movement by crawling is central to the processes of development in multicellular organisms, and is also seen in a large number of cell types in adult animals. In this chapter, we shall consider how and why cells move, but first we shall go into the processes of cell adhesion in a little more detail, a function seen in virtually all cells in multicellular organisms.

16.1.1 Cell adhesion controls the positioning of cells in tissues

In multicellular organisms, cells interact with their surroundings using a combination of direct contacts and by responding to diffusible signalling molecules. For non-motile cells, adhesion is an important function that allows them to maintain their position by direct interactions with other cells of the same type, with other cell types or with the extracellular matrix, an intricate meshwork of proteins in which cells are embedded to form tissues. Direct interactions are principally mediated by **cell adhesion molecules** (sometimes simply called adhesion molecules or abbreviated as CAMs), which interact with other molecules either expressed on the surface of other cells or embedded within the extracellular matrix.

In addition to their roles in positioning cells, cell adhesion molecules often transduce survival signals, and cells that are incorrectly positioned (i.e. cells whose cell surface adhesion molecules are not activated by their ligands) generally migrate elsewhere or may even die as a result. Indeed, for many cell types in a multicellular organism, detachment from the basal lamina or dissociation from cells that normally interact with them, leads to death by apoptosis (Chapter 14).

- ☐ Think of a reason by which survival signals triggered by adhesion molecules may also influence cell migration.
- ☒ In order to reach their final destination, migrating cells must 'survive' the journey. Particular migration pathways will provide a precise set of survival signals for different cell types.

For motile cells, cell adhesion is also crucial for movement, since migration requires the cell to carry out coordinated attachment, movement and detachment from the extracellular matrix. Migration frequently involves diffusible signalling molecules that act as guidance cues by activating receptors on the surface of the responding cell, and as a result promote movement towards, or away from, the signal.

In the earliest stages of embryonic development, virtually all cells are motile, but once they have reached their appropriate position in the developing embryo, most of them lose their motility. Nevertheless, even in the developed organism, cells still retain a sense of their correct position throughout life. In reality, the pattern of adhesion molecules expressed on different cell types determines how they will associate with each other in tissues. For example, in one early experiment, the cells of an amphibian embryo that had been dissociated and mixed in tissue culture were found to reorganize themselves to their correct arrangement—ectoderm on the outside, endoderm on the inside and elements of the mesoderm correctly associated in their right positions (the germ layers during embryonic development will be further discussed in Chapter 17). This suggests that the position of a cell is determined by two interrelated features, its state of differentiation and the set of

adhesion molecules that it expresses. Adhesion molecules of the **cadherin** family (see Section 16.2.1) are particularly important in this process—that is, the organization of tissues and maintenance of correct cell–cell contacts. Such positional adhesion usually requires that the cell participates in a number of different types of interaction. For example, epithelial cells attach both to the basal lamina and to other cells of the same type or of different types. Such cell–cell interactions are essential for the normal structuring and function of the tissue, and may be classified as **homologous** (between cells of the same type) or **heterologous** (between cells of different type).

- Give some examples of homologous and heterologous cellular interactions.
- You may have thought of many examples either from this course or from S204. Neurons connecting to each other at synapses, sheets of epithelial cells, liver hepatocytes, aggregating cells of *Dictyostelium discoideum* are all examples of homologous interactions. By contrast, heterologous interactions may include leukocytes binding to endothelium, lymphocytes binding to stromal cells in the lymphoid organs, or Schwann cells surrounding peripheral nerve axons of neurons.

Cell adhesion, or rather, dysregulated adhesive mechanisms, is also an important factor in tumour progression. A key step in the development of malignant tumours is the appearance of invasiveness, which involves two related processes. Firstly, the cancerous cells lose adhesion molecules (e.g. cadherins), so they can detach from their correct position; they then become motile, which allows them to invade normal tissue. These processes will be discussed further in Chapter 19.

16.1.2 Cell migration

Cell migration is a central event that determines pattern formation during embryonic development. The processes are extremely complex, and within this course we shall look at just a few examples (described mainly in Chapter 17). Here, it is worth mentioning that the development of the nervous system is mediated by cells migrating from the embryonic neural crest to different regions of the body in response to diffusible and contact-dependent signalling molecules, and that differentiation is induced once they reach their target site. The migration patterns of neural crest cells during embryonic development of the chick nervous system are shown in Figure 16.1. During development, it is clear that differentiation and cell adhesion are interdependent processes—expression of cell adhesion molecules and receptors on the cell surface depends on the state of differentiation; conversely, the contacts that a cell makes and the signals that it receives for survival, differentiation and division determine the genes it expresses.

In the remainder of this chapter, we shall mainly be looking at the process of migration in two model systems, and relating this to the molecules involved. The first model involves the migration of leukocytes in vertebrates, especially mammals, which has been well studied because of its medical relevance to the process of inflammation. Leukocytes can migrate from

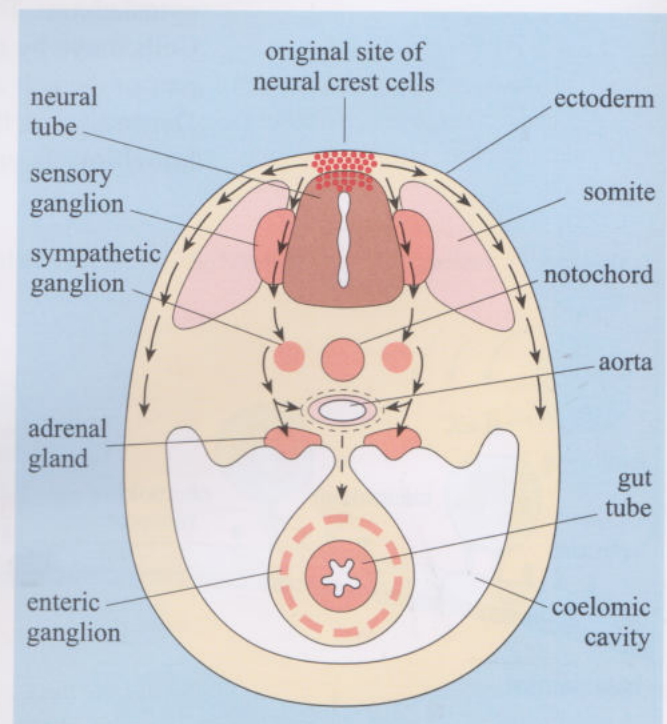


Figure 16.1 Cross-section of a chick embryo. Cells from the neural crest migrate, and differentiate into different cell types. Cells migrating below the ectoderm become pigment cells in the skin. Cells that migrate through the somites form glial cells and neurons of the sensory and sympathetic ganglia. Neurons in the enteric ganglia also come from the neural crest by migrating along the length of the body from the neck and sacral regions. Development of the nervous system (including many of the structures on this diagram) is described in detail in Section 17.3.

the blood into tissues by crossing sheets of vascular endothelial cells (Figure 16.2a). They then move through tissues; some of them return to lymphoid tissues, by crossing lymphatic endothelium to enter lymphatic vessels. Different signals direct this complex pattern of movement. The second model is the slime mould *Dictyostelium discoideum*, which has been used to examine the mechanics of cell migration. The migration of many cell types employs the same basic mechanisms for movement, even though the signals and signalling processes vary greatly between them, depending on the requirements of the organism.

Fibroblasts, osteoclasts (bone cells), endothelium and skin epithelium are other important examples of cells with potential for motility in adult vertebrates. Fibroblasts and osteoclasts continuously extend processes and migrate, remodelling connective tissues. In contrast, endothelium and epithelium are normally fixed, but following wounding, these cells can migrate rapidly into the damaged area to seal wounds (epithelium) and to create a new blood supply (endothelium). When many different cell types are placed in tissue culture, they become motile, moving across the substratum to associate with other cells. This suggests that many cell types in the adult animal have the potential to migrate, and can be induced to do so in certain physiological circumstances, such as when tissue damage occurs.

Cell migration over surfaces is a complex process, which requires coordinated control of cellular adhesion, polarization and movement, orchestrated by the cytoskeleton. To understand the processes, we first need some descriptive terms. Cells move by extending processes at the 'leading edge' (used to indicate the front part of the cell as it moves), which become attached to the extracellular matrix. Depending on their appearance, the processes are described as **pseudopodia**, **lamellipodia** or **filopodia**. Pseudopodia are stubby projections seen at the leading

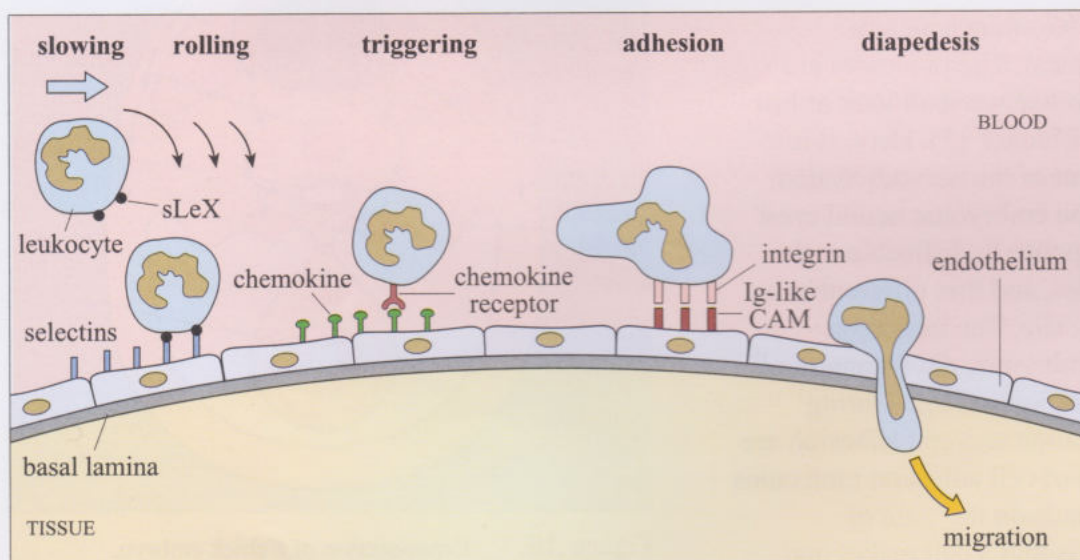


Figure 16.2 (a) A summary of the processes involved in leukocyte migration across vascular endothelium in a small vein. Leukocytes are slowed by binding to molecules on the endothelium called selectins (via interactions with ligands such as sLeX on the leukocyte surface). As they roll along the endothelium, they may be triggered by extracellular signalling molecules called chemokines, coming from the tissue or from the endothelium itself (via interactions with receptors on the leukocyte surface). Triggering activates leukocyte integrins, which can then bind to immunoglobulin (Ig) superfamily cell adhesion molecules (Ig-like CAMs) induced on the endothelial surface. This allows the cell to pull itself down into the tissues, a process called 'diapedesis'. The role of each of this group of adhesion molecules in leukocyte adhesion will be explained in detail in Section 16.2. (b) Scanning electron micrograph of a leukocyte adhering and spreading pseudopodia on a sheet of endothelial cells. (Courtesy of David Male)

edge of migrating cells such as leukocytes (Figure 16.2b) and *Dictyostelium*; typically a cell has a small number of such processes, and in time-lapse video microscopy pictures of migrating cells they give the impression of probing ahead of the cell. (See the Movie gallery section 'Cell migration and chemotaxis' on the CD-ROM.) Cells that are normally flattened in tissue culture, such as epithelia, move by extending a sheet of cytosol, a lamellipodium, at their leading edge. A very different appearance is given by filopodia, which are single filamentous extensions, produced by cells such as fibroblasts, as they extend along strands of connective tissues, for example.

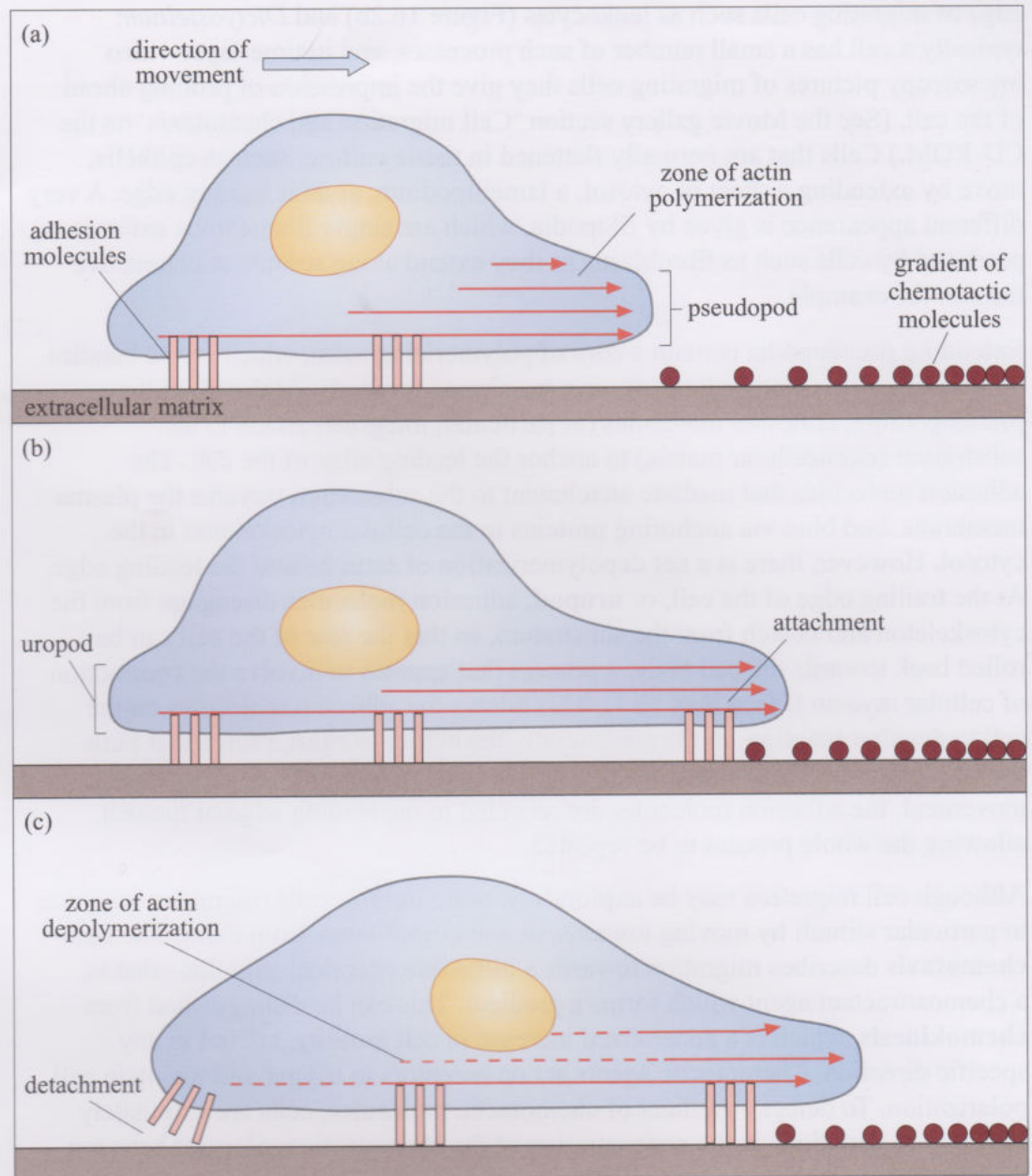
Extending pseudopodia contain a core of polymerizing actin, which forms bundles of microfilaments that push the plasma membrane forwards. At the tip of the pseudopodium, adhesion molecules (in particular, integrins) attach to the substratum (extracellular matrix) to anchor the leading edge of the cell. The adhesion molecules that mediate attachment to the substratum traverse the plasma membrane, and bind via anchoring proteins to the cellular cytoskeleton in the cytosol. However, there is a net depolymerization of actin behind the leading edge. At the trailing edge of the cell, or **uropod**, adhesion molecules disengage from the cytoskeleton and detach from the substratum, so that the rear of the cell can be rolled back towards the cell body, a process that appears to involve the contraction of cellular myosin II (see Box 12.1). This allows the adhesion molecules on the cell to develop **traction** on the substratum—the ability to exert a force that pulls the cell forwards. These processes are summarized in Figure 16.3. To maintain movement, the adhesion molecules are recycled to the leading edge of the cell, allowing the whole process to be repeated.

Although cell migration may be exploratory, more usually cells migrate in response to particular stimuli by moving towards, or sometimes away from them. The term **chemotaxis** describes migration towards a diffusible chemical stimulus—that is, a chemoattractant agent which forms a gradient. This can be distinguished from **chemokinesis**, which is a generalized increase in cell motility, but not in any specific direction. Chemotactic agents act on receptors to trigger and maintain cell polarization. To detect a gradient of chemotactic molecules, cells are exquisitely sensitive to variations in the concentration of the chemotactic molecules between their leading and trailing edges; differences of less than 2% can cause polarization. At one stage, it was thought highly improbable that gradients of chemotactic molecules could be maintained in the complex cellular environment found in tissues. However, many chemotactic molecules bind to components of the extracellular matrix, thereby forming a gradient (Figure 16.3). Directional migration across surfaces, in response to chemotactic stimuli immobilized on the substratum is termed **haptotaxis**.

The following sections use leukocyte migration as a key example of cell adhesion and migration, a subject that was introduced in S204, Book 3, Chapter 7. The descriptions of actin and myosin polymerization, and of cell migration given in that chapter may help your understanding of this chapter. Also, if you have studied S320 (Infectious Disease), you will find that the material here gives you further insight into the cell biology of the immune reactions described in S320, Book 3, Chapter 2.

Figure 16.3

(a) A cell moves by extending a pseudopod, driven by actin polymerization. (b) Adhesion molecules at the leading edge attach to the extracellular matrix, whereas those at the trailing edge (known as the 'uropod') detach from it. (c) Depolymerization of actin at the trailing edge, coupled with the overall tension in the cytoskeleton, cause the cell to move (in this example from left to right). Cells move up gradients of chemotactic molecules bound to the extracellular matrix, a process called 'haptotaxis'.



Summary of Section 16.1

- 1 Cell adhesion is a property of many cell types which position themselves by attaching to other cells or to the extracellular matrix using adhesion molecules in the plasma membrane. In addition to this function, some adhesion molecules also transduce signals from the extracellular environment to the cell.
- 2 Cell migration involves both the interaction of adhesion molecules with elements of the cytoskeleton, and the coordinated attachment, movement and detachment of adhesion molecules from the extracellular matrix. Cell migration is central to the development of eukaryotic organisms, but also occurs during tissue regeneration and repair.
- 3 Many cells have the potential for motility. The direction of cell migration is determined by chemotactic signalling molecules.
- 4 The slime mould *Dictyostelium discoideum* and mammalian leukocytes are the best-studied examples of mobile eukaryotic cells. The basic processes of motility are similar in all eukaryotes.

16.2 Cell adhesion molecules (CAMs)

In this section, we shall be examining some of the adhesion molecules that allow cells to interact with each other or with the extracellular matrix. Since adhesion molecules perform a variety of functions, it is not surprising that they belong to several different families.

What is required of an adhesion molecule depends very much on the function of the cell and its interactions. For example, an epithelial cell positioned between other epithelial cells requires adhesion molecules on the basal membrane (on the cell) to interact with the basal lamina (on the extracellular matrix), and different ones on the lateral membrane to bind to other cells. These molecules hold the cell in position. Since the epithelial cell is not motile, the adhesion molecules make relatively permanent contacts with their ligands (junctional adhesion). By comparison, a leukocyte that is migrating through a tissue binds only transiently to the extracellular matrix, and the cell must have some way of controlling the attachment and detachment of its adhesion molecules in relation to cell movement (non-junctional adhesion).

- ☐ Suggest two ways in which a cell could change its adhesive properties by modulating its surface adhesion molecules.
- ☒ A cell could change the strength of binding of individual adhesion molecules for their ligand. Alternatively, it could change the numbers of molecules present on the plasma membrane or their distribution.

We distinguish here between the **affinity**—that is, the binding strength of a single adhesion molecule for its ligand, and the **avidity** of binding, which is the overall strength of cellular adhesiveness, determined by the density and distribution of adhesion molecules in a localized region of the plasma membrane, as well as by the affinity of the individual adhesion molecules. There has been much discussion of whether cells control their adhesion by adjusting the affinity or the avidity of their adhesion molecules. In fact, both mechanisms are possible and particular adhesion molecules, such as some integrins (discussed below), can change either their affinity or their surface distribution and/or density.

Table 16.1 lists the cell adhesion molecules involved in junctional and non-junctional adhesion, and their extracellular ligands. Section 16.2 will mainly consider molecules that mediate non-junctional adhesive interactions between different cells and between cells and the extracellular matrix. Some of these molecules such as the cadherins (Section 16.2.1) and integrins (Section 16.2.2) are also involved in forming anchoring junctions (adherens junctions, desmosomes, focal adhesions and hemidesmosomes), and this will be discussed when appropriate. Others, however, such as the immunoglobulin (Ig) superfamily CAMs (Section 16.2.3) and selectins (Section 16.2.5) mostly mediate non-junctional interactions. We shall also briefly describe some of the extracellular ligands of adhesion molecules, in particular those forming the extracellular matrix (Section 16.2.4). Note that a detailed description of all molecules involved in cell adhesion (including those mediating occluding and communicating junctions) is beyond the scope of this chapter.

Table 16.1 The different types of junctional and non-junctional cell adhesive processes and molecules involved in cell adhesion.

JUNCTIONAL CELL ADHESION	Type of cell junction	Cell adhesion molecules*	Extracellular ligands*
cell–cell	tight junctions	claudins, occludin	claudins, occludin in adjacent cell
	adherens junctions	<i>cadherins</i>	<i>cadherins in adjacent cell</i>
	desmosomes	<i>non-classical cadherins</i> (<i>desmoglein</i> , desmocollin)	<i>non-classical cadherins in adjacent cell</i> (<i>desmoglein</i> , desmocollin)
	gap junctions	connexins	(connexins form channel called connexon with adjacent cell)
	chemical synapses	non-classical cadherins (protocadherins)	non-classical cadherins (protocadherins) in adjacent cell
cell–matrix	focal adhesions	<i>integrins</i>	<i>extracellular matrix proteins</i>
	hemidesmosomes	<i>integrins</i>	<i>extracellular matrix proteins</i>
NON-JUNCTIONAL CELL ADHESION			
cell–cell		<i>cadherins</i>	<i>cadherins in adhesive cell</i>
		<i>Ig superfamily CAMs</i>	<i>integrins, Ig superfamily CAMs in adhesive cell</i>
		<i>selectins</i>	<i>carbohydrate groups on glycoproteins in adhesive cell</i>
		<i>integrins</i>	<i>Ig superfamily CAMs in adhesive cell</i>
cell–matrix		<i>integrins</i>	<i>extracellular matrix proteins</i>
		membrane proteoglycans	extracellular matrix proteins and growth factors

* The molecules that will be considered in some detail in this chapter are given in italics.

16.2.1 Cadherins

The integrity of tissues, both during development and in the adult animal, depends on homologous adhesion between cells, a process that is principally mediated by proteins of the cadherin family. In vertebrates, cadherins are classified according to where they were first identified; for example, E-cadherin was identified on epithelial cells, whereas VE-cadherin is found on vascular endothelial cells. In practice, individual cadherins are not always confined to a single cell type, and most cells express more than one cadherin. Cadherins and their cytoskeleton-anchoring proteins **catenins**, are widely conserved throughout the animal kingdom. For example, a single cadherin gene is found in *C. elegans*, which is spliced in one of two ways to make one cadherin that functions in neurons and another that functions in epithelial cells. However, in vertebrates the cadherin family is much larger, with more than 30 different types of cadherin having been generated by gene duplication.

- Can you suggest a reason to explain, at least partly, why there are many more different types of cadherin in vertebrates compared with *C. elegans*?
- The function of cadherins is to allow homologous interactions between cells, often but not exclusively in mature junctions (adherens junctions and desmosomes). As there are many more types of differentiated cell (and also organs and tissues) in a vertebrate than in *C. elegans*, you may have thought that a larger number of members of the cadherin family would be required to form different types of cell–cell adhesions in vertebrates.

Homologous and heterologous interactions refers to adhesion between cells, whereas homophilic and heterophilic interactions refers to binding between adhesion molecules of the same kind or of a different kind, respectively, in the same cell or in adjacent cells.

Cadherins are single-pass transmembrane molecules that interact with identical cadherins on other cells via the distal N-terminal domain of their extracellular region (distal, in this context, means furthest away from the plasma membrane). The interaction has been likened to a zipper, and the binding between cadherins on adjacent cells is dependent on Ca^{2+} ions (Figure 16.4). Molecular models of some cadherins show them to have a binding pocket in the N-terminal domain, which can accept a residue from a neighbouring cadherin. The pocket is formed by a group of three amino acid residues (His–Ala–Val), and this motif is found in all major cadherins. Mutating this region or interfering with the binding of these residues causes the cadherins to lose their adhesion. The regions around the binding pocket are also important, because they determine the specificity of the interaction, so each cadherin only binds its own kind (homophilic binding).

Cadherins also cluster together on the surface of each individual cell. The current model of cadherin adhesion envisages cadherins interacting laterally with neighbouring cadherins on the same cell by Ca^{2+} -independent interactions, whereas, as explained in the previous paragraph, head-to-head interactions between cadherins on apposing cells are Ca^{2+} -dependent. In reality, each cadherin dimerizes with a neighbour on the same cell, forming homodimers.

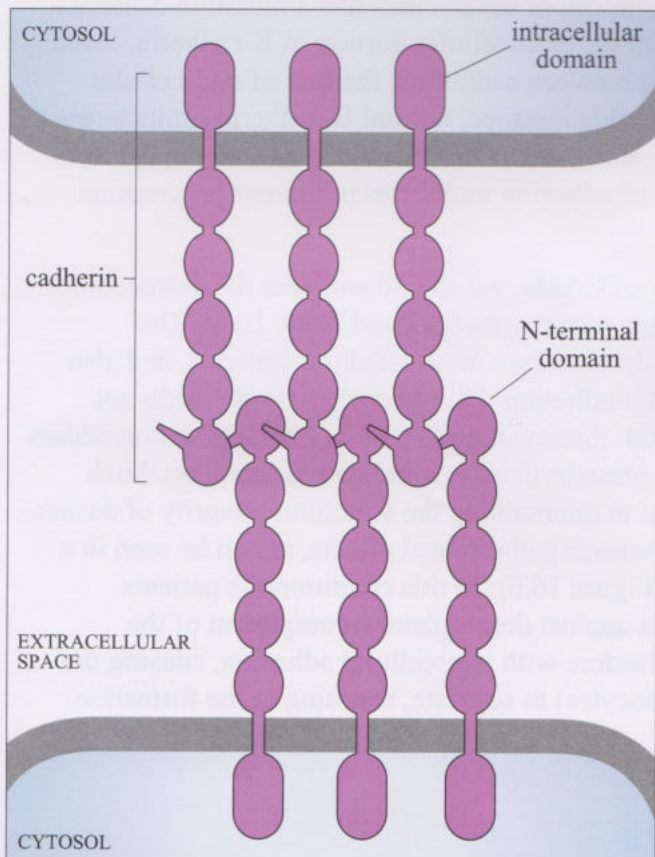


Figure 16.4

A model for the interaction of two apposing cells mediated by cadherins. Residues from one cadherin enter a pocket on a cadherin molecule of a neighbouring cell. Multiple interactions between individual cadherin molecules make a zipper-like connection between the two cells. The model shows three cadherin molecules on each cell, although many more molecules would normally be involved. More detailed models show that each cadherin dimerizes with a neighbour on the same cell, which would produce extensive areas of adhesion, extending into the plane of the diagram.

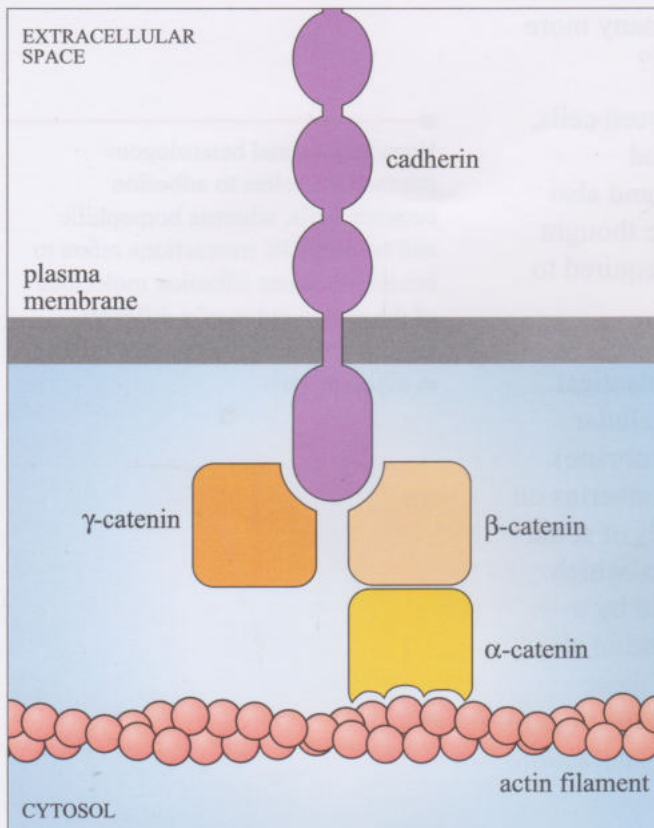


Figure 16.5 Cadherins interact with actin filaments via catenins, which act as cytoskeletal anchoring proteins. Binding of the intracellular domain of cadherin to β -catenin brings α -catenin-bound actin filaments to the adherens junction. As you will learn in Chapter 17, β -catenin has another important role in intracellular signal transduction, especially during development. Another protein called γ -catenin (or plakoglobin) regulates cadherin function.

Multiple interactions between homodimers result in extensive areas of adhesion between cells (forming a continuous adhesion belt in some cell types, such as epithelial cells).

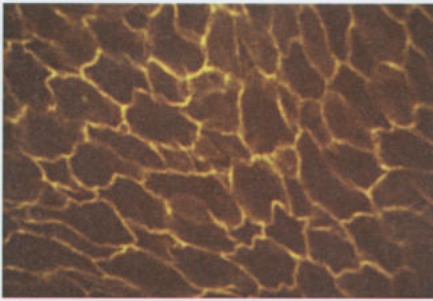
Interestingly, the extracellular domains in the cadherins are structurally similar to the extracellular domains found in another large family of cell adhesion molecules (the Ig superfamily of CAMs), which are important in heterologous cell adhesion, and are described in Section 16.2.3.

The intracellular domain of the cadherins interacts with a set of three proteins, called catenins, which link the adhesion molecule to cytoskeletal actin filaments (Figure 16.5). The importance of catenins in cadherin-mediated cell interactions is demonstrated by experiments in which catenin–cadherin interactions have been disrupted. In vertebrates, the deletion of catenin genes or deletion of the sequence encoding the intracellular domain of cadherins weakens homologous cell–cell adhesion, which demonstrates that it is necessary for cadherins to interact with the cytoskeleton in order to develop their full function. The zones of cadherins with catenins and actin filaments form the adherens junctions between cells referred to in Chapter 6 (see Figure 6.35).

Changes in cadherin function are important in a number of diseases. Dysregulated cell adhesion is a key factor in tumour progression. In order to invade tissues, malignant tumours must first detach from their correct position. Loss of cadherins and of homologous cell adhesion as a result, is associated with the development of malignancy in a number of tumour types. For example, in one study of a particular type of breast cancer it was found that four out of seven cases had a mutation causing a deletion of part of the extracellular portion of E-cadherin, leading

to disruption in homophilic binding between cadherins; the loss of intercellular adhesion facilitated invasiveness. In this instance, normal E-cadherin limits tumour spread, and E-cadherin is therefore sometimes referred to as a tumour suppressor. You will learn more about the role of adhesion molecules in tumour progression in Chapter 19.

Before leaving the cadherin family of CAMs, we should consider the desmosome, another form of intercellular junction (see Figure 6.35 and Table 16.1). The transmembrane proteins that form desmosomes are related to cadherins, and also mediate Ca^{2+} -dependent intercellular adhesion. However, these proteins do not interact with microfilaments but with intermediate filaments (this interaction occurs via a set of cytoskeletal anchoring proteins that are unrelated to catenins). Such junctions are particularly important in maintaining the structural integrity of tissues. Disrupting desmosomes can have serious pathological effects, as can be seen in a human disease called pemphigus (Figure 16.6). In this condition the patients produce an autoantibody that reacts against desmoglein, a component of the desmosome. The autoantibodies interfere with intercellular adhesion, causing the epidermal cells of the skin (keratinocytes) to separate, resulting in the formation of blisters.



(a)



(b)

Figure 16.6

(a) Immunofluorescence used to demonstrate antibodies in the skin of a patient with pemphigus vulgaris. Autoantibodies are bound to desmoglein, a component of the desmosome which is seen around the edge of keratinocytes. (b) Clinical appearance of a patient with pemphigus vulgaris, showing extensive blistering and erosion of the skin.

16.2.2 Integrins

Integrins are a large and widely distributed family of adhesion molecules found in multicellular animals, including vertebrates, nematodes (e.g. *C. elegans*) and insects (e.g. *Drosophila*). Even sponges have integrins, and this has led to the view that the evolution of integrins was an essential step in the development of multicellular animals; they are absent from plants, fungi and bacteria. Integrins are involved in both intercellular adhesion (non-junctional cell adhesion) and the interactions of cells with the extracellular matrix (focal adhesions and hemidesmosomes, and non-junctional cell adhesion). We shall examine the integrins in some detail, partly because of their importance in cell migration, and partly because of the instructive character of their structure–function relationship.

Integrins are heterodimers formed by two non-covalently associated chains, one α chain and one β chain, both of which traverse the cell membrane with their C-termini lying in the cytosol (Figure 16.7a). A large number of different chains have been described (and classified according to numbers such as $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$, $\beta 2$, etc., or to letters referring in some instances to the cells they were first described in, such as αL for lymphocytes or αM for monocytes/macrophages). Many of the chains can associate with more than one of the other type to produce a variety of integrins with different ligand-binding properties (Table 16.2). Note that integrin ligands are not integrins but other molecules (heterophilic binding). It is also worth mentioning that some integrins bind to more than one ligand, via different binding sites. In this section, we shall focus on the $\beta 2$ integrins, which

are expressed on leukocytes and have important roles in leukocyte adhesion and migration (Figure 16.2). Although β_2 integrins mediate cell–cell adhesion rather than cell–matrix interactions (these are mainly mediated by β_1 integrins; see Table 16.2), the same principles in terms of structure and activity apply for all integrins.

Table 16.2 Integrins of the β_1 and β_2 families.*

Integrin name(s)	Polypeptides	Ligands
VLA-1 [†]	$\alpha_1\beta_1$	collagen
VLA-2	$\alpha_2\beta_1$	collagen
VLA-3	$\alpha_3\beta_1$	collagen, laminin, fibronectin
VLA-4	$\alpha_4\beta_1$	fibronectin, vascular cell adhesion molecule-1 (VCAM-1)
VLA-5	$\alpha_5\beta_1$	fibronectin
VLA-6	$\alpha_6\beta_1$	laminin
LFA-1	$\alpha_L\beta_2$	intercellular adhesion molecules ICAM-1, ICAM-2, ICAM-3
CR3/Mac-1	$\alpha_M\beta_2$	ICAM-1, complement fragments C3b and C4b
CR4/gp150, 95	$\alpha_X\beta_2$	C3b, C4b
—	$\alpha_D\beta_2$	unknown

* You do not have to learn all the integrins or their ligands listed here.

[†] VLA = very late antigen, so called because some of these molecules appear on lymphocytes late after activation. LFA-1 = lymphocyte functional antigen-1. $\alpha_M\beta_2$ was first identified as a complement receptor termed CR3, and independently identified as a marker of macrophages (Mac-1). CR4 is also a receptor for complement fragments. CR4 is also a receptor for complement fragments and was originally termed glycoprotein, with two chains of M_r 150 000 (α) and 95 000 (β).

- ☐ An integrin is a CAM that mediates cell migration. With what intracellular structure would you expect the intracellular portion of the integrin to interact?
- ☒ The cytoskeleton.

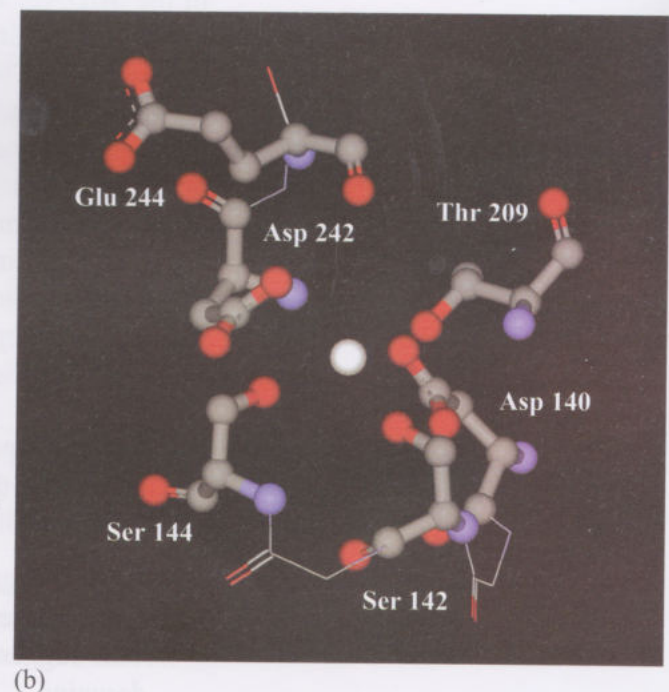
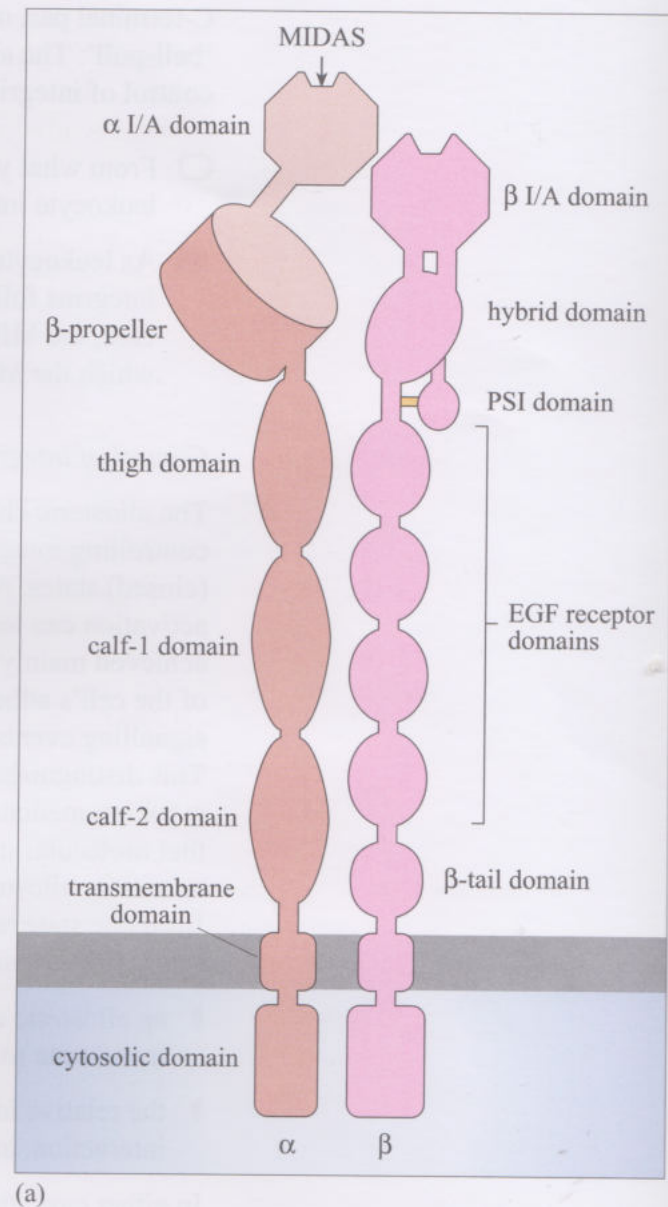
All integrins except one bind to actin-based microfilaments. (The one exception binds to intermediate filaments.) By cross-linking the extracellular ligand with the intracellular cytoskeleton, integrins allow a cell to gain traction on the extracellular matrix or on other cells.

The binding of integrins to their ligands (their affinity) requires extracellular divalent cations (either Ca^{2+} or Mg^{2+} depending on the integrin), and this is reflected in the structure of their extracellular regions. The extracellular portion of both chains is folded into domains, shown diagrammatically in Figure 16.7a for $\alpha_M\beta_2$ integrin ($\alpha_M\beta_2$ is found on some populations of leukocytes, including macrophages). An area of the so-called I/A domain in the N-terminus of the α chain is critical for the adhesion function of this integrin. It contains a metal ion coordination site, formed by residues from three adjacent polypeptide loops (serine, threonine and aspartate residues shown in detail in Figure 16.7b). The crystal structure of this domain

Figure 16.7 (a) A diagrammatic representation of the domains in an integrin. The α chain has a distal I/A domain with a metal ion-dependent adhesion site (MIDAS); this is linked to a β -propeller, a cylindrical structure containing seven strands of β pleated sheet. Three domains separate this section of the α chain from the transmembrane domain and cytosolic domain. The distal portion of the β chain contains an I/A domain and a hybrid domain formed by the polypeptide chain folding back on itself. The N-terminus of the β chain is located in the small PSI domain, which forms a disulfide link (yellow) with a lower part of the β chain. This structure is supported on four EGF (epidermal growth factor) receptor-like domains and a small β -tail domain. The polypeptide traverses the plasma membrane, and the C-terminus is located in the cytosolic domain. Some integrins lack the I/A domain of the α chain, in which case ligands appear to interact with the I/A domain of the β chain and the β -propeller of the α chain. (b) The molecular model shows the six amino acid residues surrounding the MIDAS in the I/A domain of the α chain of $\alpha_M\beta_2$ integrin. The central Mg^{2+} ion is shown in white. Note how the positively charged Mg^{2+} ion is coordinated by the electronegative, polar oxygen atoms (red) of hydroxyl groups (Ser or Thr) or carboxylate groups (Asp or Glu). (Based on pdb file 1id0.)

was solved in 1995, and showed that other residues are important for metal ion coordination. Specifically, it was observed that a glutamate residue from elsewhere in the structure formed part of the sphere of residues surrounding the metal ion. The significance of the finding that a glutamate residue could interact with Mg^{2+} was immediately apparent. It had long been known that integrin ligands contained a conserved aspartate or glutamate residue, and it was suspected that the carboxylate group of these amino acids coordinated with a metal ion. It has since been shown that integrin ligands bind the I/A domain via this **metal ion dependent adhesion site (MIDAS)**. By accommodating aspartate or glutamate residues, the MIDAS of integrins allows their binding to specific ligands. For example, $\alpha_M\beta_2$ (also known as CR3 or Mac-1; see Table 16.2) recognizes and binds to the group of residues Arg–Gly–Glu (RGE) on its main ligand, an adhesion molecule of the Ig superfamily called ICAM-1 (Section 16.2.3). Many other ligands of integrins contain either an RGD or an RGE tripeptide motif at their integrin binding site. Note that when we mention the I/A domain in the following paragraphs, we shall usually be referring to that of the α chain, even though some integrins lack this I/A domain. In this case, ligands appear to interact with the I/A domain of the β chain and the β -propeller of the α chain.

Even more interesting is the way in which binding of the ligand to the MIDAS can be modulated. The site itself can either be ‘open’ and accessible to the coordinating carboxylate group of the Asp or Glu residues on the ligand, or ‘closed’, in which case it cannot bind to ligands. The switch from the closed to the open form is accompanied by shifts in the polypeptide loops surrounding the MIDAS, which affect the way in which the metal ion is coordinated. This in turn relates to longer-range allosteric changes, which extend throughout the I/A domain, and include a 1 nm downward shift of a section of the α helix near the



C-terminal part of the domain—a segment that we shall subsequently refer to as the ‘bell-pull’. The significance of the ‘bell-pull’ segment in ligand binding and in the control of integrin activation is discussed further below.

- ☐ From what you have learnt, what is the possible functional significance of a leukocyte integrin with a MIDAS that can either be in an open or closed state?
- ☒ As leukocytes move, they must first attach to the substratum by activating integrins following interaction with proteins in the extracellular matrix (in which case, the MIDAS should remain open). Cells then move, and finally detach (for which the MIDAS should be closed).

Control of integrin activation

The allosteric changes in the I/A domains of integrins provide a means for controlling integrin activation by switching between active (open) and inactive (closed) states. As we shall see later, intracellular signalling events as a result of cell activation can lead to changes in cell adhesion and integrin binding, which is achieved mainly by means of changing the conformation of the extracellular portion of the cell’s adhesion molecules. This is referred to as **inside-out signalling**, where signalling events occurring within the cell affect what occurs on the cell surface. This distinguishes it from conventional outside-in signalling (Chapter 13), such as cytokine-mediated induction of cell division. For inside-out signalling, it is thought that molecular interactions concerning the intracellular regions of integrins result in necessary allosteric changes in the extracellular I/A domain of the α chain to switch from one state of integrin activation to another. However, it is not yet known how this inside-out signal is transmitted. There are two broad possibilities:

- ☒ an allosteric change is transmitted through the domains of the α chain across the membrane to the MIDAS;
- ☒ the relative location of the α chain and β chain changes, thereby affecting their interaction, and inducing allosteric changes in the α chain affecting the MIDAS.

In either case, the allosteric changes in the α chain induce the opening and closing of the MIDAS. These possibilities are not mutually exclusive, but evidence from genetically engineered cells suggests that an interaction between the cytosolic domains of the two chains is important for integrin activation. When the cytosolic domains of either α or β integrins (or both) are removed using recombinant DNA technology, transfection of cells with these mutant forms results in constitutively active integrins. By contrast, further experiments showed that if the cytosolic domains were mutated so that the α and β chains could not be separated, the resultant integrin was inactive. This latter result indicates that when integrins are inactive, the cytosolic domains of the two chains remain together. Another observation supports this: when integrins bind to their ligands, it has been found that the cytosolic domains of the two chains separate from each other.

- ☐ After interpreting these two sets of findings to relate the activation state of the integrin to the conformation of its cytosolic domains, can you suggest a model for controlling integrin activation?
- ☒ When integrins are bound to their ligands (I/A open), the cytosolic domains are separate, and when they are inactive (I/A closed), the domains are together. This suggests that a cell could activate its integrins by separating their cytosolic domains.

The cytosolic domains of many integrin β chains contain a motif that can interact with a number of molecules that associate with the cytoskeleton. One such protein called talin binds to integrins, resulting in their conversion into the high-affinity form. This led to the idea that a cell can control the activation of its integrins by binding cytoskeleton-associated proteins that cause the separation of the α and β chains (Figure 16.8). We shall return to this subject in Section 16.4, but here we shall continue by looking at how the altered orientation of the integrin chains could cause the change in the I/A domains.

We referred to the C-terminal helix ‘bell-pull’ adjoining the I/A domain, and the way in which this moves down when the integrin binds to its ligand. In accordance with this observation, we have seen that if the α helix of the bell-pull segment is pulled down, the ligand binding site in the I/A domain will open to accommodate the ligand. These observations led to the proposal that the allosteric changes running through the integrin molecules which mediate activation of integrins are a result of the downward displacement of the bell-pull segment. Since the β chain also has an I/A domain with a bell-pull segment, it is thought that a similar mechanism could mediate an allosteric relay in the β chain. One piece of evidence supporting this model is that an antibody that recognizes an activated form of an integrin binds to the bell-pull in the β chain, which suggests that the allosteric changes affect both chains.

Integrins as signalling molecules

In the description above, we regarded integrins as having the single function of cell adhesion whose activation is controlled by inside-out signalling. In fact, many integrins are also involved in conventional outside-in cell signalling and, like growth factor receptors, their binding to ligands can lead to changes in gene expression and other cellular responses such as differentiation and cell survival. For example, integrin binding to its ligand can activate the PI 3-kinase pathway and, ultimately, inhibit apoptosis.

- ☐ Relate the ability of integrins to inhibit apoptosis to one of the functions of cell adhesion molecules described in Section 16.1.
- ☒ Cell adhesion is necessary for the survival of some types of cell.

Hence integrins transduce signals across the cell membrane. This can occur in either direction: binding of an extracellular ligand can lead to activation of intracellular signalling pathways; conversely binding of an intracellular molecule such as talin can lead to activation of the extracellular ligand-binding site and modulate adhesion. Seen in this way, integrins are molecules that can bind ligands that are either inside the cell (e.g. talin) or outside the cell (e.g. collagen), both of which are capable of passing allosteric changes across the cell membrane.

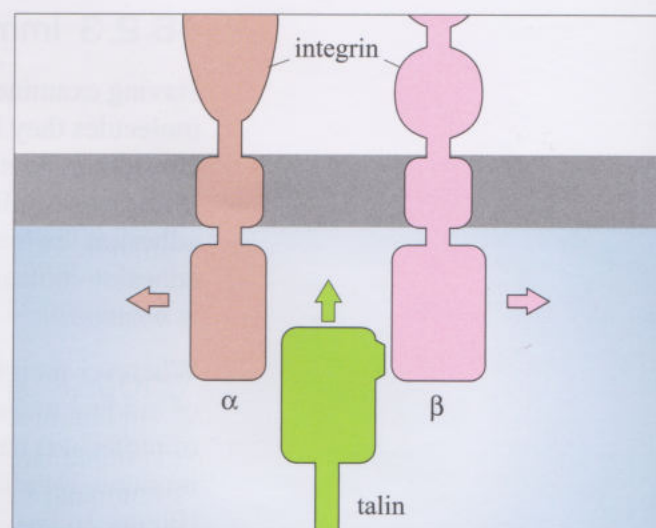


Figure 16.8 A hypothetical model of integrin activation. The head region of talin binds to the cytosolic domain of the integrin β chain, forcing the α chain and β chain to separate. This leads to allosteric changes in the extracellular domains of the integrin, which ultimately causes the MIDAS to open.

Molecular modelling 8

Go to the Study Skills file: *Molecular modelling 8*. The aim of this investigation is to relate the functions of integrins as adhesion molecules to their molecular characteristics, and to see exactly how they interact with extracellular ligands such as collagen.

16.2.3 Immunoglobulin superfamily of cell adhesion molecules

Having examined integrins in some detail, we are now going to look at some of the molecules they ligate. In addition to extracellular matrix molecules (which we consider in Section 16.2.5), several integrins bind to molecules that belong to the **immunoglobulin (Ig) supergene family**, and in this way mediate cell–cell adhesion via heterophilic interactions. In some instances, such as with neural cell adhesion molecule (N-CAM), Ig superfamily CAM-mediated binding between cells is *homophilic*.

Whatever their binding partners, the activity of Ig superfamily CAMs is independent of binding to Ca^{2+} (or Mg^{2+}) in contrast with cadherins and integrins. This family of molecules have one or more domains of the type that were first identified in immunoglobulins (antibodies), and so they are called Ig superfamily domains (Figure 16.9a). The domain is characterized by two layers of β pleated sheet forming a barrel-shaped structure. A very large number of cell surface molecules belong to this family, but we are principally concerned here with integrin ligands such as intercellular adhesion molecule-1, ICAM-1 (Figure 16.9b), which binds to $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins, ICAM-2, which binds to $\alpha_L\beta_2$ integrin (and to $\alpha_M\beta_2$ weakly), vascular cell adhesion molecule (VCAM), which binds to $\alpha_4\beta_1$ integrin. These molecules are all expressed on the surface of endothelium, where they may bind to integrins on leukocytes. Many Ig superfamily CAMs are often induced in areas of inflammation, and their main function is to mediate the migration of leukocytes from the blood into the tissues (Figure 16.2).

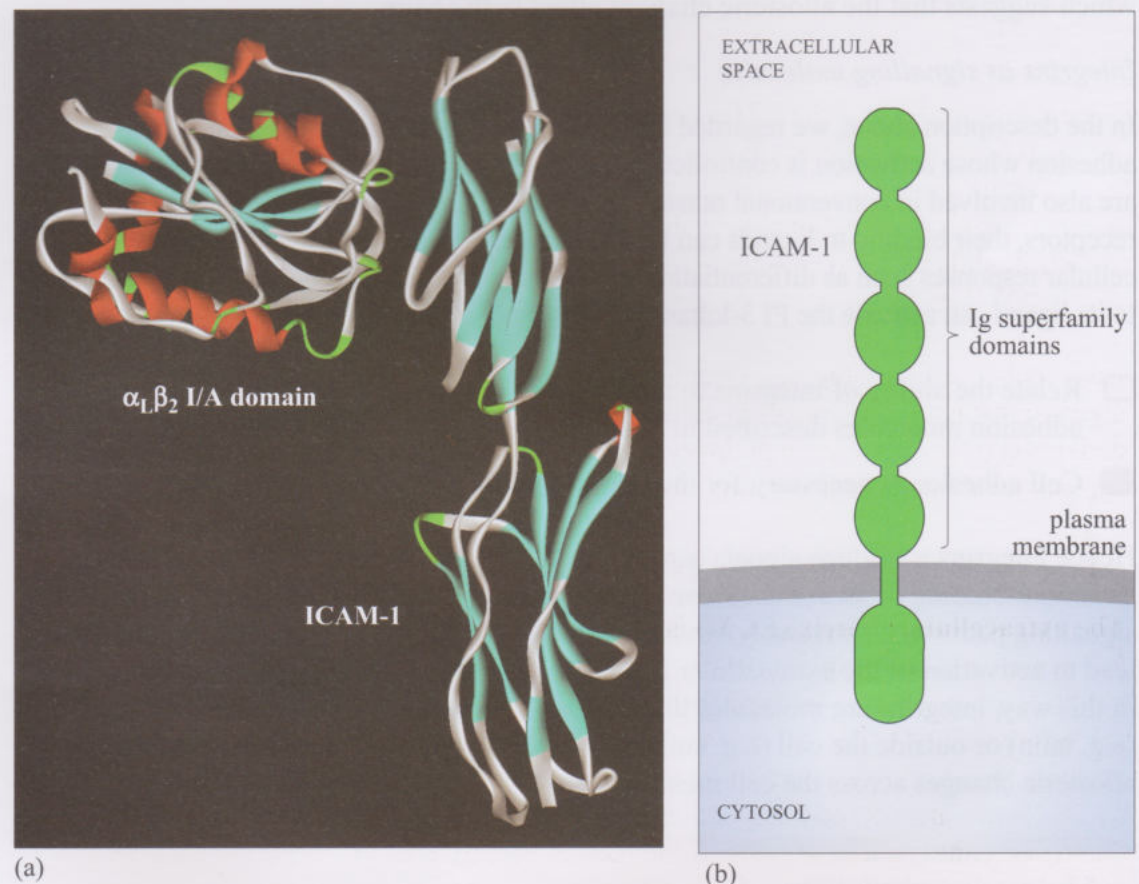


Figure 16.9 (a) Molecular model of the binding of the I/A domain of the α_L chain of $\alpha_L\beta_2$ integrin to the N-terminal domain of ICAM-1. Only two of the five Ig superfamily domains of ICAM-1 are shown in this diagram. (b) Schematic diagram of ICAM-1 showing its five immunoglobulin (Ig) supergene family domains.

The file 1MQ8-monomer.msv shows the two N-terminal domains of ICAM-1 binding to the I/A domain of the $\alpha_L\beta_2$ chain of $\alpha_L\beta_2$ integrin. The original pdb file 1MQ8 shows a dimeric form of the two molecules. One half of the structure has been hidden in the msv file given here.

The amino acid sequence of the second Ig domains of human ICAM-1 and ICAM-2 is given below. The sequence homology between ICAM-1 and ICAM-2 is around 35%, which is apparent especially when some gaps are placed in the ICAM-1 sequence. Both of these domains contain the binding site for the $\alpha_M\beta_2$ integrin.

	1	11	21	31	41	51	61																																																						
ICAM-1	L	T	L	R	C	Q	V	E	G	G	A	P	R	A	N	L	T	V	V	L	L	R	G	E	K	E	L	K	R	E	PA	V	G	E	P	A	E	V	T	T	T	V	L	V	R	R	D	H	H	G	A	N	F	S	C						
ICAM-2	F	T	I	E	C	R	V	P	T	V	E	P	L	D	S	L	T	L	F	L	F	R	G	N	E	T	L	H	Y	E	T	F	G	K	A	A	P	A	P	Q	E	A	T	A	T	F	N	S	T	A	D	R	E	D	G	H	R	N	F	S	C

- ☐ What is the consensus sequence in integrin ligands that integrins bind to.
- ☒ RGD or RGE (Section 16.2.2).
- ☐ Identify the sites on ICAM-1 and ICAM-2 that bind to the integrin, assuming that both ICAM-1 and ICAM-2 bind to $\alpha_M\beta_2$ in a similar way.
- ☒ Integrins bind to ligands containing either an RGD or an RGE tripeptide motif at their integrin binding site. The binding site on ICAM-1 is the sequence RGE as stated above (at positions 22–24), and a similar sequence RGNE is seen at the same position in ICAM-2 (at positions 22–25). One can therefore infer that this is also the binding region on ICAM-2 for the integrin, with the glutamate residue (E) entering the MIDAS. Molecules with limited homology in their primary sequence may still have functional homologies. Note that the binding site for the integrin $\alpha_M\beta_2$ and that for $\alpha_L\beta_2$ shown in Figure 16.9a are in different Ig domains of ICAM-1, the second and the first, respectively.

Like cadherins and integrins, engagement of some Ig superfamily CAMs can activate signal transduction pathways in cells, and effect several cellular responses (e.g. activation of several kinase pathways following recruitment of endothelial ICAM-1 by leukocyte integrins is essential for leukocyte adhesion to endothelial cells).

16.2.4 Proteins of the extracellular matrix

The **extracellular matrix (ECM)** is material secreted by cells and laid down in the extracellular space. It includes polysaccharides called glycosaminoglycans (GAGs), which form chains that are usually bound to certain proteins, in this case called ‘proteoglycans’. GAGs and proteoglycans form a hydrated gel, within which fibrous proteins, including collagen, fibronectin and laminin, are embedded. The composition of the ECM varies greatly, depending on the tissue and its function. Here, we shall mostly be concerned with some of the protein components of the ECM that act as substrata to which integrins attach. Adhesion to ECM components via integrins (Table 16.2) is essential, both for allowing cells to move through tissues and for positioning cells within the body. Indeed, attachment to the ECM and spreading on it is required for the survival of some cell types, a phenomenon called **anchorage dependence**, in which cell survival signals are transduced via the integrins, as noted above.

The fibrous protein **collagen** is located in both basal laminae and connective tissues, where it is a structural component. There are different types of collagen, encoded by different genes; they each have a triple-helical structure, but the composition of the polypeptide chain varies.

- ☐ What types of integrin would you expect to find located on the basal plasma membrane of endothelium or epithelium interacting with collagen?
- ☒ $\beta 1$ integrins such as VLA-1, which allow the cell to bind to collagen and other components of the basal lamina, are selectively located on the basal plasma membrane of endothelium or epithelium.

Another important component of the ECM is the protein **fibronectin**. This molecule exists in many different forms, including a water-soluble form found in the blood, which modulates blood clotting, as well as the insoluble forms found in tissues.

- ☐ There is only one gene for fibronectin. How is it possible to have numerous forms of a protein, when there is only one gene encoding it?
- ☒ You may have thought of alternative splicing, with different combinations of exons used in each form of the protein (see Figure 10.38).

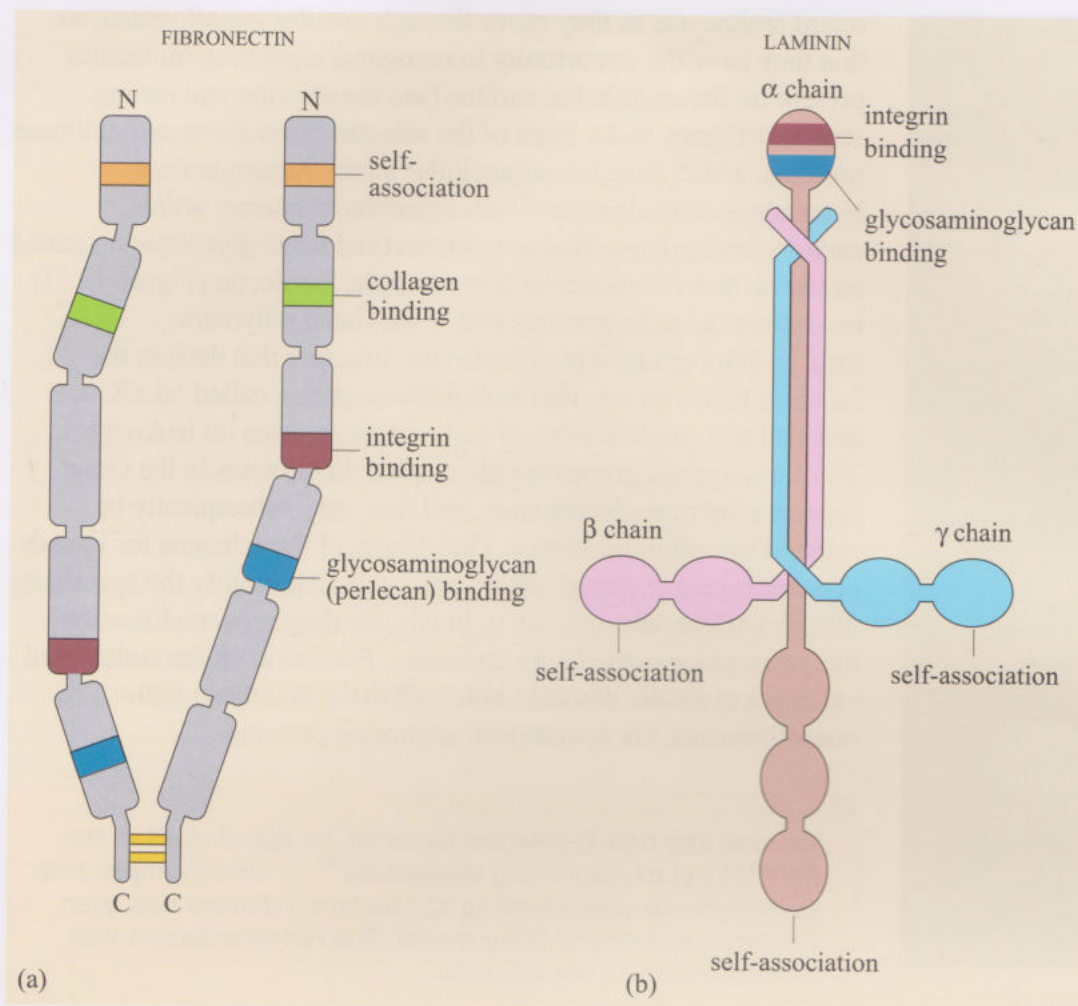
In fact, the fibronectin gene is made up of approximately 50 exons. The many different forms of the protein are made by alternative splicing of a single transcribed pre-mRNA (Section 10.6.1). Fibronectin is a dimeric modular protein, consisting of numerous fibronectin repeat domains, a structure that is found in a large number of other molecules. Each region of fibronectin, and each domain within it, has specific functions, including self-association, binding to collagen and attachment to cells (Figure 16.10a).

You can see an example of fibronectin domains in file 1FNA-RGD.msv using ViewerLite™. In this example, we have highlighted an RGD sequence, located on an external loop of the fibronectin domain. This representation is derived from the file 1FNA.pdb.

- ☐ What can you predict about the function of the RGD sequence in a fibronectin domain?
- ☒ The RGD motif is that found in binding substrates for integrins. A fibronectin domain containing such a sequence is the cell-binding domain, as it will directly interact with integrins expressed on the cell surface, such as $\alpha_4\beta_1$ and $\alpha_5\beta_1$ (Table 16.2).

Laminin is another major component of basal laminae (Figure 16.10b). This branched molecule forms a network that cross-links and stabilizes the basal lamina, and binds to $\alpha_6\beta_1$ integrin on cells. Laminin is particularly important during early development, where it is a major component of the ECM, and acts as a guide for migrating cells that are growing cellular processes. For example, as the axons of developing neurons grow out to find their targets, they attach to laminin in order to extend (Chapter 17).

We have highlighted here just some of the structural components of the ECM, because of their ability to bind to $\beta 1$ integrins and thus support cell migration. Of the other molecules associated with the ECM, several are proteoglycans, having a

**Figure 16.10**

Structural proteins of the basal lamina. (a) Fibronectin is a dimeric molecule, cross-linked by disulfide bridges (yellow), in which the chains are usually not identical, but result from alternative mRNA splicing, such that the two chains usually have some domains in common. Each domain has distinctive binding properties. (b) Laminin is a trimeric molecule formed of α , β , and γ chains. As there are several chains of each type, a large variety of different laminins can be formed. The molecule forms a felt-like network by self-association between the different arms of the cross-shaped molecule. It also binds glycosaminoglycans (e.g. perlecan), and has an integrin-binding site.

protein core with glycosaminoglycan side-chains, which are frequently sulfated, added to the OH groups of specific serine or threonine residues. Proteoglycans also serve an important role in cell migration because many extracellular signalling molecules, including chemokines, attach to them (Section 16.5.1). By immobilizing extracellular signalling molecules in this way, gradients of chemotactic molecules can become established in tissues, which, in association with the structural components of the ECM, act as trackways for migrating cells.

Proteoglycans are also present on the surface of different cells types. They include the family of transmembrane molecules called **syndecans** (the transmembrane core protein is bound to several glycosaminoglycan chains on the extracellular side, whereas their intracellular regions interact with the cytoskeleton). Such molecules serve to immobilize chemotactic molecules on the surface of endothelium, thereby allowing them to signal to circulating leukocytes. Syndecans also bind to growth factors, and act as adhesion molecules, so they also have a key role in wound healing.

16.2.5 Selectins and sialoadhesins

Adhesion molecules such as integrins interact with protein ligands, but there are also numerous examples of adhesion molecules that bind to carbohydrate residues. Such proteins are described as **lectins**, and here we shall look at one particular family of such molecules, the **selectins**, which are involved in leukocyte migration from blood into tissues. There are three types of selectin: E-selectin is principally found on endothelial cells; P-selectin is found on platelets and endothelial cells; and L-selectin is found on lymphocytes. The principal function of these molecules is to

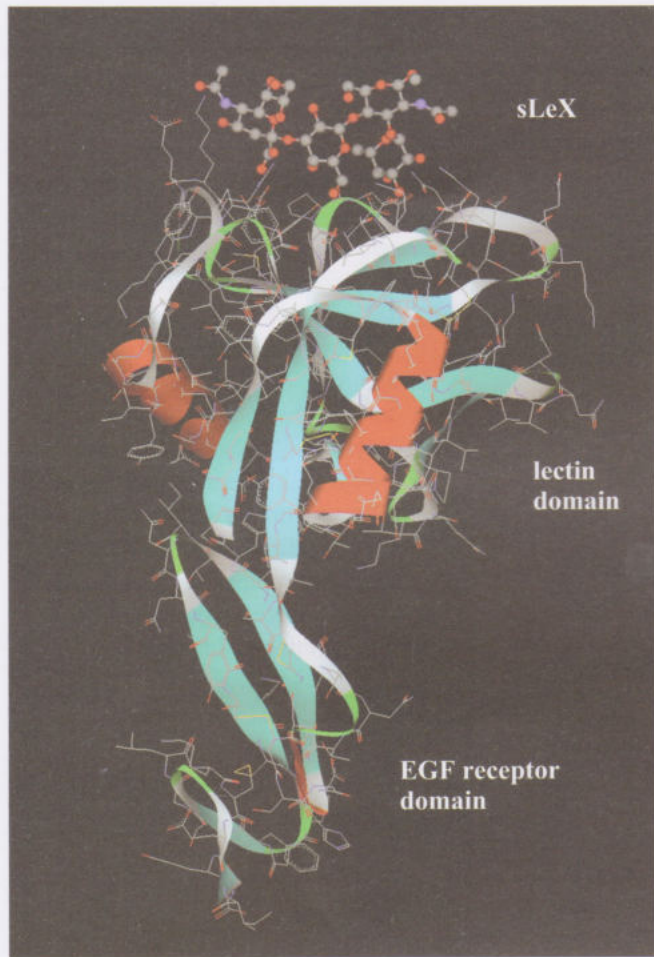


Figure 16.11 The two N-terminal domains of E-selectin, with the carbohydrate sialylated Lewis X ligand (sLeX) in the binding site on the lectin domain.

retard leukocytes as they move through venules (small veins), so that they have the opportunity to recognize signalling molecules present on the endothelial surface (see the slowing and rolling stages of Figure 16.2). Each of the selectin types are transmembrane proteins, which have an extracellular lectin domain at their N-terminus. This domain allows selectins to interact with carbohydrate groups on glycoproteins (and some glycolipids) located on the surface of other cells. For example, E-selectin (Figure 16.11) on endothelial cells interacts with a sialylated polymeric carbohydrate group, which is also the structure that defines the Lewis X blood group. This carbohydrate group, called 'sLeX' is attached to a small number of cell surface proteins on leukocytes. The carbohydrate groups are attached to the proteins in the Golgi apparatus by transglycosidases, and they may subsequently be sulfated by sulfotransferases. The affinity of the selectins for ligands depends on the extent of sulfation, so cells can modify the specificity of their surface selectins, according to the degree of modification that takes place in the Golgi apparatus. E-selectin on the endothelial cell has a cytosolic domain, which allows it to interact with microfilaments, via cytoskeletal anchoring proteins.

You can see how E-selectin binds to its ligand sLeX in the file 1G1T-sLeX.msv using ViewerLite™. In this example, the carbohydrate group binding to the lectin domain has been rendered in a space-filling model. The representation was derived from the file 1G1T.pdb.

A related family of molecules, the **sialoadhesins**, are also heavily sialylated. Examples of these molecules are found on leukocytes, and more widely on cells in the nervous system. Because these cell surface molecules are usually large and strongly negatively charged, they tend to prevent other cells from approaching and interacting (unless these other cells have specific receptors for the sialoadhesin, or, alternatively, the sialoadhesin-expressing cell interacts with other cells via other pairs of adhesion molecules).

16.2.6 Different types of adhesion molecule have distinctive functions

Look back at Figure 16.2, where the different stages of leukocyte migration across endothelial cells are described. The interaction of selectins with their sialylated ligands, and of integrins with Ig superfamily CAMs, both occur during leukocyte migration. However, they have fundamentally different roles. Selectins are important in the initial stages of leukocyte adhesion. The affinity of selectins for their ligands is quite low, so binding of leukocytes to the endothelium is transient, unless additional adhesion molecules are recruited. In contrast, integrins can bind to Ig superfamily CAMs quite firmly, when in a high-affinity conformation, so that the leukocyte can adhere strongly to the endothelial cell (how leukocytes then migrate through or in between the endothelium, and the molecular mechanisms mediating this event, are still not completely understood). Hence the different functions of the adhesion molecules in cell migration can be related to the different nature of their molecular interactions.

Summary of Section 16.2

- 1 Adhesion molecules allow cells to attach to each other or the substratum or extracellular matrix.
- 2 Cadherins mediate cell–cell junctional adhesion via homophilic interactions, and are associated with the cytoskeleton via anchoring proteins called catenins.
- 3 Integrins are very important in controlling cell adhesion, as their affinity (strength of binding to a ligand) may be modulated by inside-out signalling. The metal ion dependent adhesion site on the I/A domain of integrins can be present in an open or closed form, which determines whether the integrin can bind to its substrate(s).
- 4 Different integrins can bind to components of the extracellular matrix or adhesion molecules on other cells. Some integrins also transduce survival signals for anchorage-dependent cells.
- 5 Ig superfamily CAMs contain several extracellular Ig domains. By contrast with integrins and cadherins, binding of Ig superfamily CAMs to their ligands is independent of Ca^{2+} (or Mg^{2+}).
- 6 Collagen, fibronectin and laminin are important components of the ECM and basal laminae, and bind to different $\beta 1$ integrins.
- 7 Integrins, Ig superfamily cell adhesion molecules (Ig superfamily CAMs) and selectins are all involved in leukocyte migration into tissues, each type of molecule having a different function in the process. Selectins bind to carbohydrates on glycoproteins to tether migrating cells, whereas the interaction between integrins on the leukocytes and Ig superfamily CAMs on the endothelial cells allows the leukocyte to migrate through the endothelium.

16.3 Control of cell adhesion in cell migration

The control of cell adhesion is vital during cell migration: the allosteric modulation of integrin affinity is clearly well suited to a process in which cells need to attach and detach rapidly from the substratum. However, there are several other ways, in which cells can change their adhesive properties by modulating the expression of cell adhesion molecules at the cell surface or, in other words, by changing their avidity as follows:

- changing the total number or density of adhesion molecules on the cell surface (1 and 2 in Figure 16.12);
- changing the grouping of the molecules on the plasma membrane (3 in Figure 16.12);
- shedding or internalizing adhesion molecules (4 and 5 in Figure 16.12).

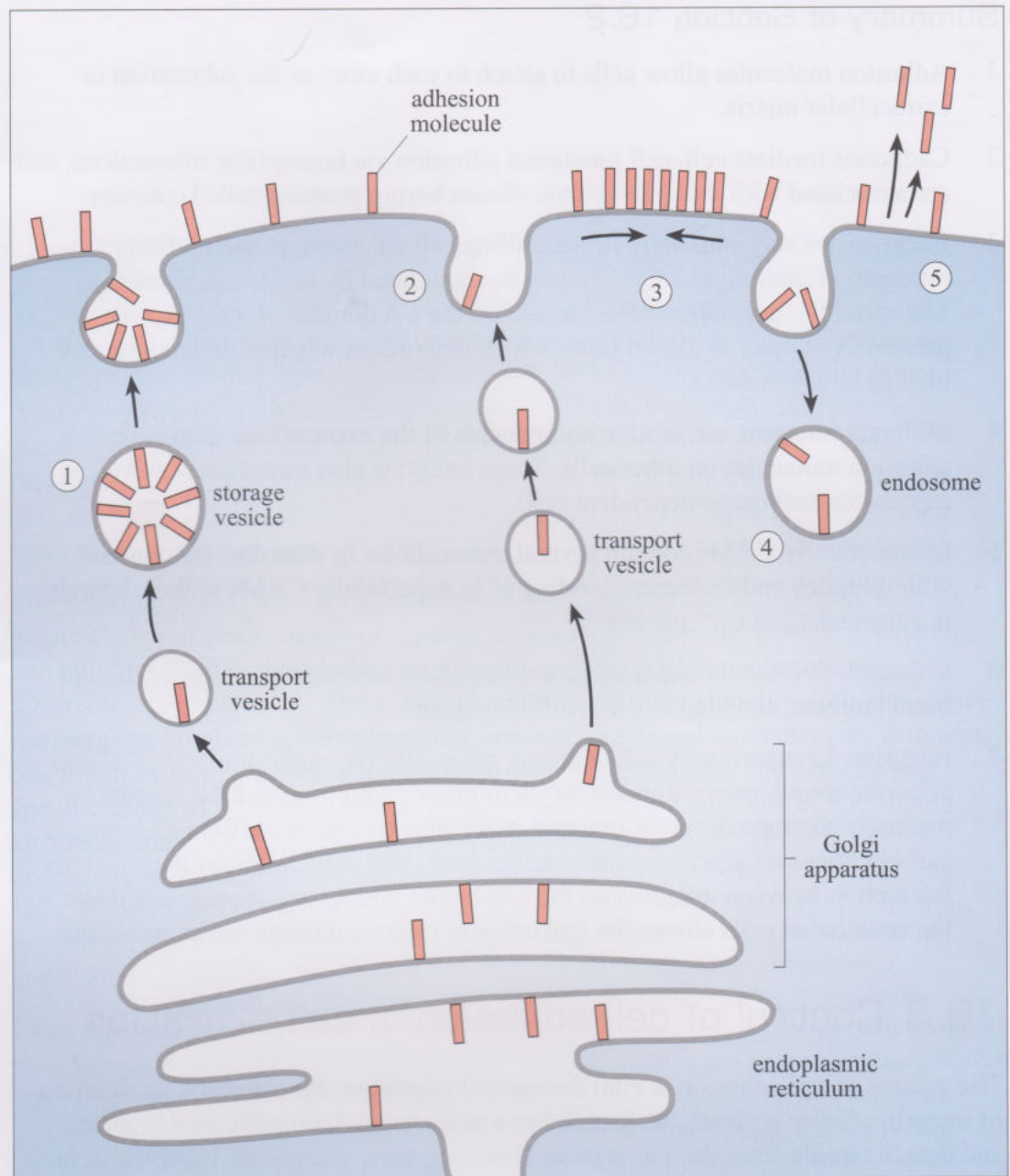
These three different ways of controlling cell adhesion are summarized in Figure 16.12 and discussed in the following three sections.

16.3.1 Cell-surface expression of adhesion molecules

The level of expression of molecules such as ICAM-1 and E-selectin can be changed by one of two mechanisms. Molecules can be released to the plasma membrane from intracellular stores, or expression can be increased by new synthesis (see 1 and 2, respectively, in Figure 16.12).

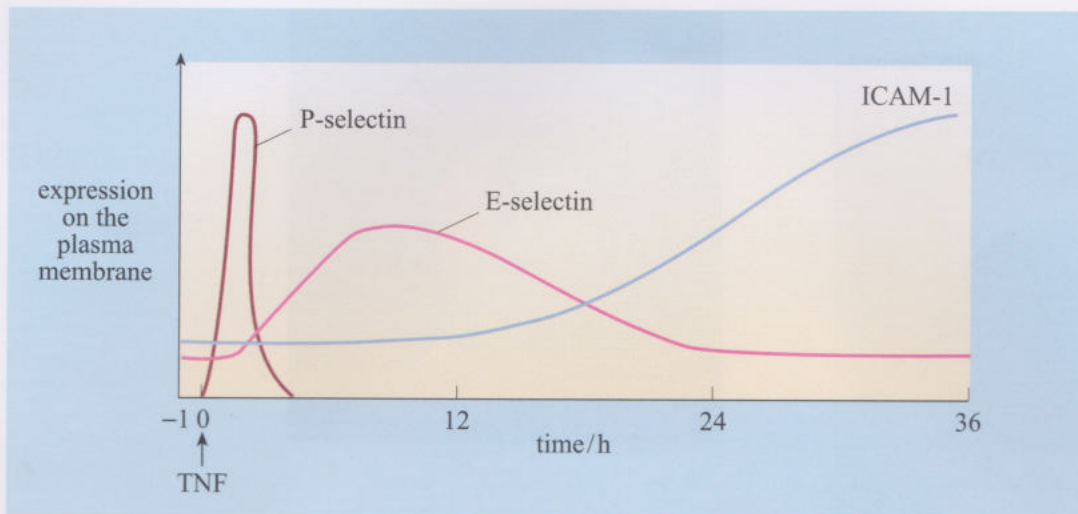
Figure 16.12

Control of adhesion molecule expression at the cell surface. Adhesion molecules that are constitutively synthesized can be stored in vesicles, such as the Weibel Palade body of endothelium, and then rapidly released on the cell surface, following an appropriate signal (1). Induced synthesis of new molecules and expression on the cell surface takes several hours (2). Clustering of molecules on the cell surface can create high-avidity patches (3). Adhesion molecule expression is reduced either by re-internalization via endosomes (4) or by release from the cell surface (5).



- ☐ In terms of controlling adhesion, how would you expect these two mechanisms to differ?
- ☒ Release of molecules from intracellular stores can take place quite rapidly and induce swift changes in a cell's adhesive properties, whereas adhesion modulated by increased protein synthesis proceeds more slowly, as synthesis of new molecules usually takes several hours or days.

These effects are illustrated by the changes in adhesion molecule expression that occur on endothelial cells during inflammation or damage to blood vessels. The cytokine tumour necrosis factor (TNF) is released at sites of inflammation and/or injury such as a wound, and can activate endothelium to promote leukocyte adhesion or the binding of platelets. Figure 16.13 shows how TNF affects the expression of adhesion molecules on cultured endothelial cells, and illustrates how control is exerted by these two different mechanisms, release from intracellular stores and *de novo* synthesis. P-selectin has an important role in blood clotting, and needs to be quickly expressed on the apical (blood side) surface of the endothelium

**Figure 16.13**

Induction of adhesion molecules on the surface of microvascular endothelium in culture following stimulation with tumour necrosis factor (TNF) indicated as time 0.

to facilitate platelet aggregation and prevent haemorrhaging. In order to achieve this, it is principally released from intracellular stores, showing an early but transient expression on the surface of the endothelium. By contrast, E-selectin is synthesized early after stimulation with TNF, although some is stored. Therefore its expression on the plasma membrane is somewhat later and more lasting than that of P-selectin. ICAM-1 is normally expressed on endothelium, but synthesis gradually increases over 12–48 hours after stimulation. Remember from Figure 16.2 that endothelial selectins (such as E-selectin) and Ig superfamily CAMs (such as ICAM-1) are involved in different aspects of leukocyte adhesion. E-selectin mediates rolling, which precedes strong adhesion mediated by the interactions between ICAM-1 and leukocyte integrins.

- ☐ Can you envisage any physiological reason why P-selectin induction should be faster than E-selectin induction?
- ☒ P-selectin has a role in blood clotting: it is important that this occurs quickly after blood vessels are damaged. E-selectin is involved in the migration of leukocytes into tissues in inflammation. Inflammation is a more protracted type of response.

16.3.2 Surface grouping of adhesion molecules

Even before it was appreciated that adhesion molecules could change their affinity by inside-out signalling, it was already thought that changes in the adhesive properties of cells were produced by clustering of adhesion molecules into high-avidity patches on the plasma membrane. Measurements of the affinity of individual adhesion molecules for their substrates shows that they are generally quite low, and it is only by binding to many adhesion molecules that significant adhesion develops. Observations of leukocytes interacting with endothelium, and of other cell types migrating over a substratum, support the concept of high-avidity patches. Cell adhesion molecules on leukocytes are clustered in patches located on the tips of microvilli, directed towards the endothelial cell (Figure 16.14a). Adhesion molecules and many receptors become polarized on migrating cells (Figure 16.14b). In cells interacting with the ECM, adhesion is also localized in ECM–cell interacting hotspots: such patches are called **focal adhesions**. These adhesions are characteristic of many cell types, and they mark the positions where bundles of actin filaments

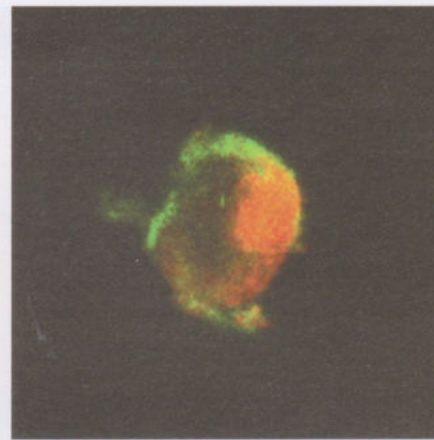
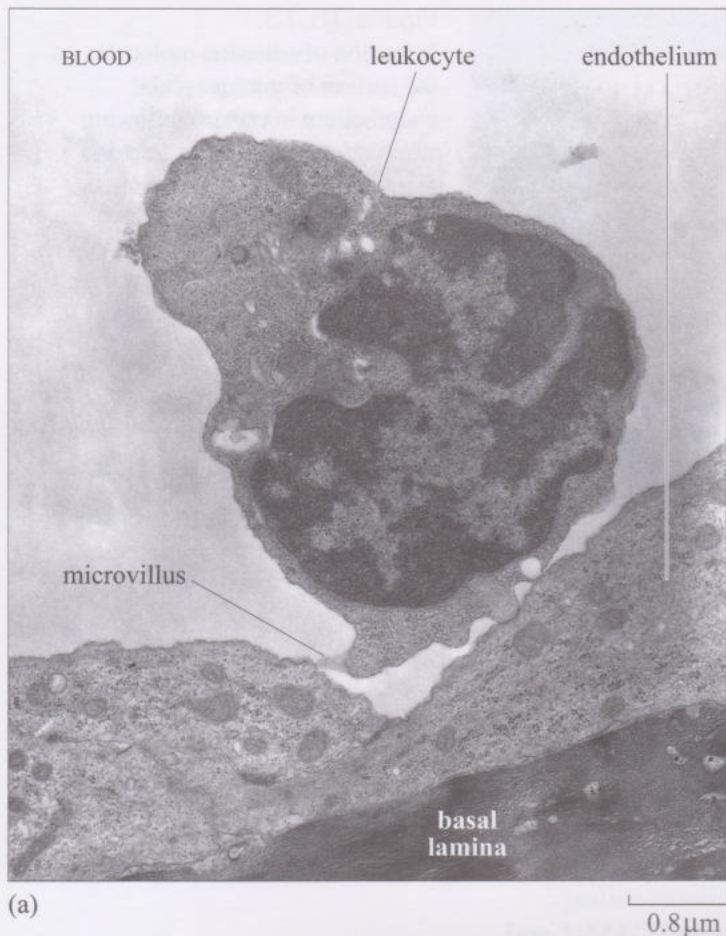


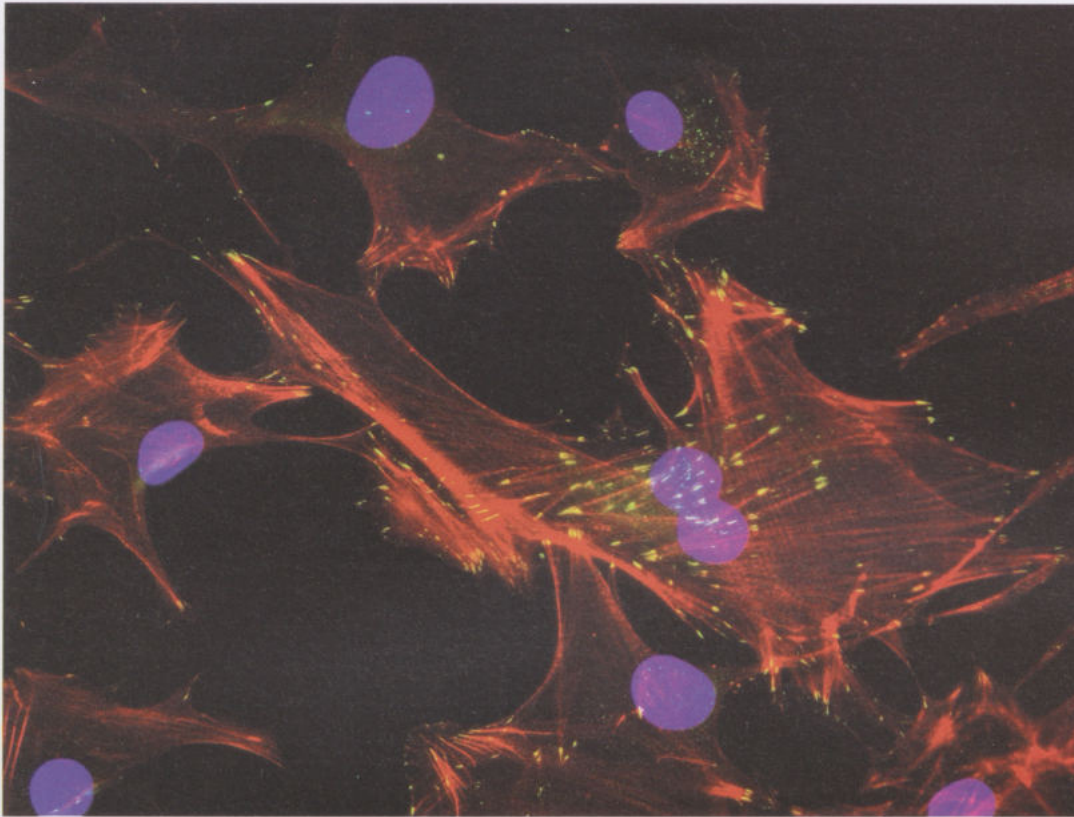
Figure 16.14 (a) Transmission electron micrograph of a leukocyte binding to an endothelial cell. Note that adhesion is localized to small patches, and is associated with the electron density around the plasma membranes of both cells. (b) Immunofluorescence showing clustering of the $\alpha_4\beta_1$ integrin polarized towards the migrating pseudopod in a chemokine-activated monocyte (a type of leukocyte). The cell has been labelled to show an integrin ($\alpha_4\beta_1$) in green and the receptor (CXCR4) for the activating chemokine in red. Integrins and chemokine receptors are distributed uniformly in unstimulated cells. Following activation with a chemokine, these molecules distribute to different areas of the plasma membrane of the migrating cell. (Courtesy of Dr E. Solito)

forming stress fibres terminate, and where clusters of integrins are located on the cell surface. The tyrosine kinase **focal adhesion kinase (FAK)** is also localized at these points, and is thought to activate components of the adhesion complex, to reinforce the contact point (Figure 16.15). FAK activity is sensitive to the composition of the extracellular matrix, so it may also be involved in transducing survival signals to anchorage-dependent cells. The signalling mechanisms that lead to the formation of focal adhesions will be considered in Section 16.5.

The reorganization of leukocyte integrins into patches follows the activation of these cells by chemotactic signals; however, the effect is selective. For example, activation of neutrophils (a type of leukocyte) with the chemotactic cytokine interleukin-8 (IL-8) causes rapid clustering of $\alpha_L\beta_2$ integrin into patches, but not of $\alpha_M\beta_2$ integrin. (Another example of integrin clustering on activated leukocytes is shown in Figure 16.14b.) If the integrin now engages ICAM-1 on the endothelial cells, this will lead to clustering of several ICAM-1 molecules on the endothelial surface (in this way, ICAM-1 functions as an enzyme-associated receptor since clustering triggers association of the intracellular domain with signalling molecules with enzymatic activity). If the leukocyte does not bind to an endothelial cell, the high-avidity patches disperse.

16.3.3 Limiting cell interactions

Loss of adhesion molecules from the cell surface occurs via two routes. Cell adhesion molecules can be internalized, typically following phosphorylation by specific kinases and accumulation in clathrin-coated pits. Alternatively, cell

**Figure 16.15**

Stress fibres in an astrocyte identified by fluorescent phalloidin, a protein extracted from a mushroom that binds specifically to F-actin (red). Focal adhesion kinase (FAK) is identified by immunofluorescence with an antibody conjugated with a fluorescent molecule (green), and cell nuclei are stained with a blue fluorescent dye (DAPI) that binds to DNA. (Courtesy of Christine Lancashire)

adhesion molecules may be enzymatically cleaved and shed from the cell surface. For example, leukocytes may shed L-selectin once they have crossed the endothelium. Since selectins are involved in slowing leukocytes rolling along the endothelium within the bloodstream, they are not required once the cells have entered the tissues. By comparison, integrins play an important role both in the adhesion between leukocytes and the endothelium, and in the subsequent movement within tissues (involving interactions with other cells and the ECM); they are not shed from the cell surface.

Soluble adhesion molecules shed from the plasma membrane could inhibit the binding of adhesion molecules to their ligands by sequestration (Section 13.2.3), and it has been proposed that this could be a physiological way of preventing leukocytes binding to endothelium (it has even been suggested that soluble adhesion molecules may become the basis for therapies targeted to inflammatory processes).

- ☐ How effective do you think soluble adhesion molecules such as ICAM-1 would be at interfering with the binding of leukocytes to endothelium?
- ☒ Because the affinity of individual adhesion molecules for their ligands is relatively low, one would not expect them to be very effective, as they would be competing with cell surface adhesion molecules organized into high-avidity patches.

Summary of Section 16.3

- 1 Cells can regulate the functional activity of their adhesion molecules by changing the surface density or distribution of the molecules (avidity), and/or by modulating their affinity.
- 2 Adhesion molecules tend to cluster in high-avidity patches (which are called 'focal adhesions' in cell-matrix interactions).

- 3 Adhesion can be rapidly modulated by changing the affinity of the individual adhesion molecules. Alternatively, adhesion can be downregulated by internalizing or shedding adhesion molecules from the cell surface.

16.4 Cell movement

In the following sections, we shall look at some of the mechanisms that control cell movement and chemotaxis. These mechanisms have been studied in *D. discoideum*, an organism that proliferates as amoeboid single cells. Under appropriate environmental stimulation the amoeboid cells migrate towards each other and aggregate to form a multicellular 'slug', which ultimately differentiates into a fruiting body. Even in the absence of chemotactic stimuli, the cells of *Dictyostelium* (and leukocytes) show polarity, with F-actin concentrated in the leading pseudopod and myosin II in the uropod (Section 16.1.2). (Many of the studies on *Dictyostelium* have used extracellular cAMP as the chemotactic stimulus, whereas studies with leukocytes generally use chemokines.)

You can see how leukocytes and *Dictyostelium* are attracted to chemotactic molecules in the movies 'Chemotaxis of neutrophils' and 'Chemotaxis of *Dictyostelium*' located in the Cell migration folder of the *Movie Gallery*.

16.4.1 Actin polymerization underlies pseudopod extension

The basic mechanisms for migration are very similar in all cell types, and depend on actin polymerization. Actin filaments are seen in a number of different forms in migrating cells:

- 1 At the leading edge of the advancing pseudopod, the filaments push forward in branched arrays.
- 2 Along the sides of cells, the filaments form bundles of stress fibres, which help to maintain the integrity of the cell and prevent it spreading as it moves (Figure 16.15). The stress fibres are arranged parallel to the direction of movement or tension acting on the cell.
- 3 In other areas, small gel-like networks of actin fibres form, with no particular orientation relative to cell movement.

At this point, you may find it helpful to review Section 12.1.2, which describes how actin filaments extend by polymerization, a process that underlies the functions of actin in cell movement.

- ☐ What class of molecule stabilizes actin filaments by binding to their plus or minus ends? What protein cross-links actin filaments into stress fibres?
- ☒ Capping proteins bind to the ends of actin filaments; α -actinin cross-links stress fibres.

In order to extend pseudopodia during migration, a cell of *Dictyostelium* or a neutrophil assembles actin filaments at the leading edge, a process that requires nucleation of new filaments, thereby allowing the rapid growth of actin and the formation of branches in extensive networks. In the advancing pseudopod, nucleation is mediated by two actin-related proteins, ARP2 and ARP3, which, together with a number of other proteins, form the **ARP complex** (Figure 16.16). ARP2 and ARP3 are evolutionarily very ancient, and have about 45% structural homology with actin itself. The ARP complex preferentially nucleates actin polymerization when it is attached to the side of a pre-existing actin filament. The new branches develop at an angle of 70° to the original filament, thus generating a branched network of filamentous actin. The rate of polymerization of the actin filaments is determined by the relative activities of two other proteins, **thymosin**, which inhibits filament polymerization, and **profilin**, which enhances it (Figure 16.16a). These two proteins compete with each other for the pool of free actin monomers within the cell, and thereby affect the rate of addition of actin monomers to the plus end of the growing filament. Cells may prevent the further extension of actin filaments by the addition of capping proteins (Figure 16.16b) to the plus end. Indeed, most of the filaments in a stationary cell are normally capped in this way.

The minus end of an actin filament may remain attached to the ARP complex, which prevents depolymerization. However, filaments generally detach from their nucleating proteins, and are therefore susceptible to degradation from the minus end. Also, another protein, **cofilin**, attaches to the sides of the filaments causing strain, and promoting depolymerization of the filament. Since cofilin preferentially binds to actin-ADP (as opposed to actin-ATP), it promotes dissociation of the minus end of the filaments. Immunofluorescence studies of actin and cofilin in migrating cells show that actin polymerization occurs at the leading edge (be it pseudopodia, lamellipodia or filopodia, depending on the cell type), whereas cofilin is behind this edge, where there is a greater proportion of actin-ADP (Figure 16.17).

Actin-ATP is often referred to as the T-form and actin-ADP as the D-form of actin.

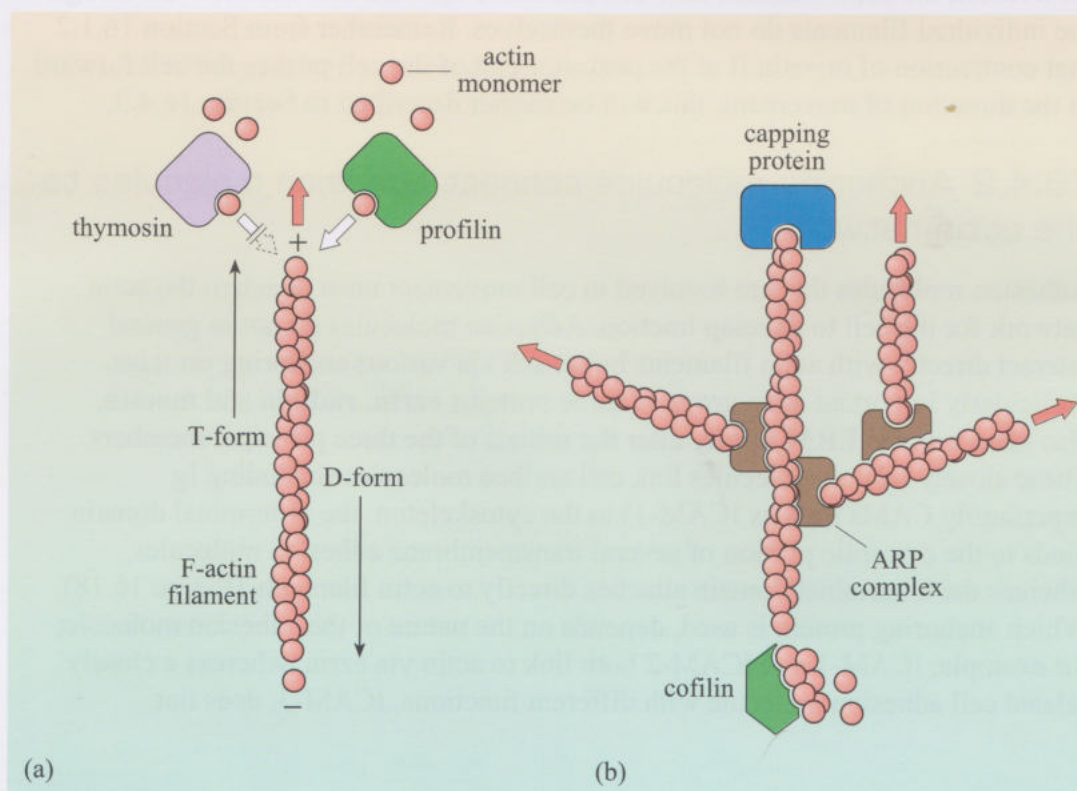
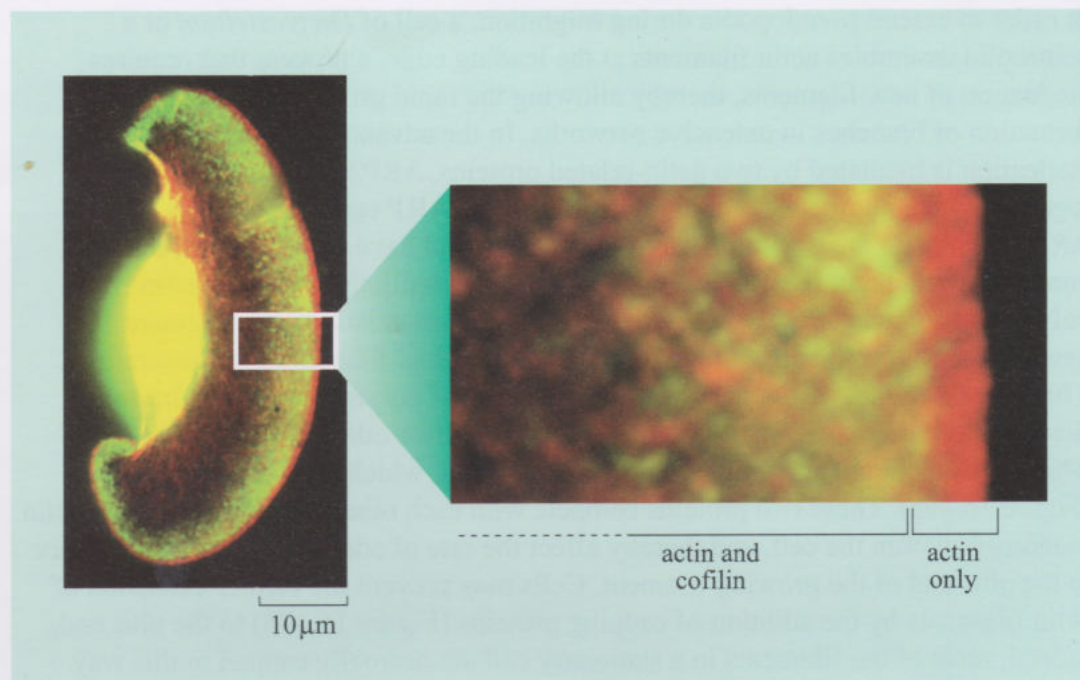


Figure 16.16

(a) Polymerization of an actin filament takes place preferentially from the plus end (+), and is promoted by the transfer of actin monomers from the cytosolic pool by the protein profilin. Thymosin limits the rate of growth of the filament by sequestering actin monomers so they cannot attach to the plus end. As actin hydrolyses its bound ATP, there is a progressive increase in the D-form of actin in the filament. (b) In an advancing pseudopod, a branched network of filaments forms. Capping proteins may attach to the plus end of the filament to limit its further extension, but the attachment of the ARP complex to the sides of the filament nucleates a new filament. The protein cofilin promotes depolymerization of the filaments behind the leading edge, by attaching to the side of older filaments.

Figure 16.17

Location of F-actin (red) and cofilin (green) identified by immunofluorescence in the lamellipodium of a migrating keratocyte. Areas where the F-actin and cofilin are colocalized appear yellow. F-actin polymerization occurs throughout the lamellipodium, whereas cofilin is preferentially localized behind the leading edge. (Data from T. Svitkina and G. Borisy, 1999)



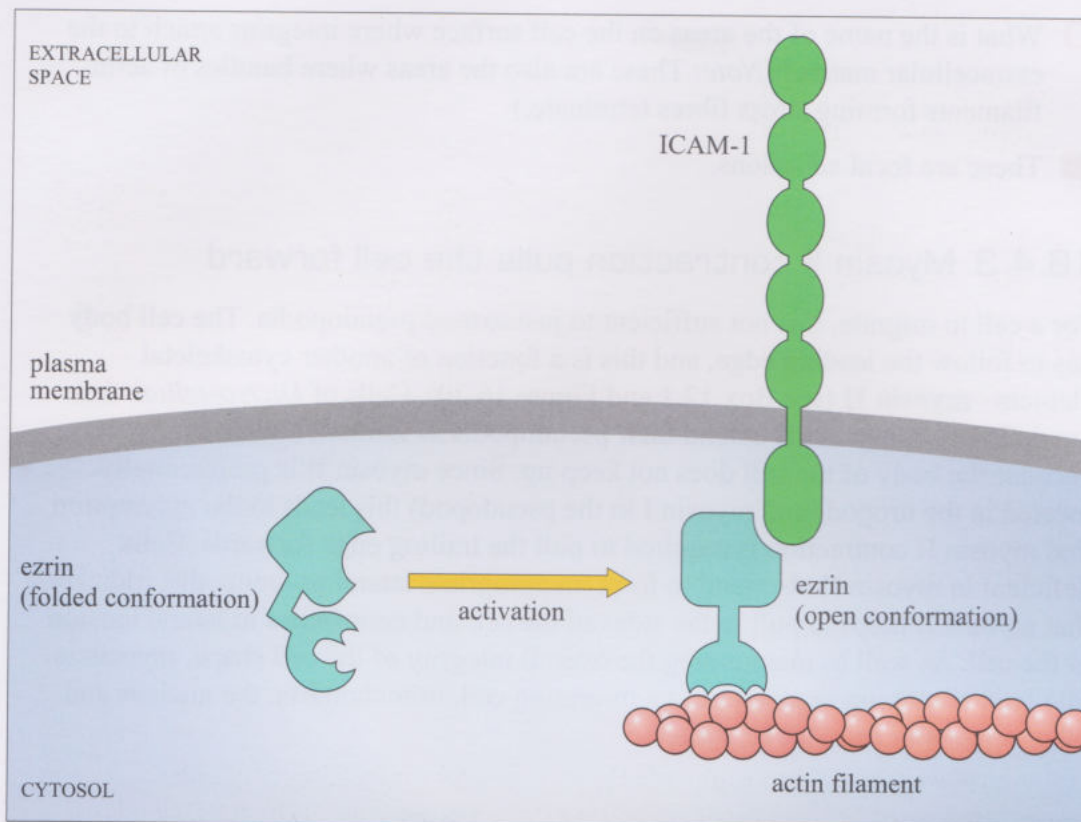
In *Dictyostelium* and neutrophils (as well as in other migrating cells), the consequences of the processes regulating the actin network at the leading edge detailed here are:

- 1 A net assembly of new actin filaments and branching of the actin network at the leading edge of the pseudopod, which pushes the plasma membrane forward.
- 2 A net disassembly of old actin filaments in the zone behind the leading edge at the rear.

As a result, the actin filament network can move forward as a whole, even though the individual filaments do not move themselves. Remember from Section 16.1.2 that contraction of myosin II at the posterior end of the cell pushes the cell forward in the direction of movement; this will be further described in Section 16.4.3.

16.4.2 Anchoring molecules connect adhesion molecules to the actin network

Adhesion molecules that are involved in cell movement must attach to the actin network for the cell to develop traction. Adhesion molecules do not in general interact directly with actin filaments but attach via various anchoring proteins. Particularly important are a group of three proteins **ezrin**, **radixin** and **moesin**, also known as the **ERM family** after the initials of the three principal members. These closely related molecules link cell surface molecules (including Ig superfamily CAMs such as ICAM-1) to the cytoskeleton: the N-terminal domain binds to the cytosolic portion of several transmembrane adhesion molecules, whereas the C-terminal domain attaches directly to actin filaments (Figure 16.18). Which anchoring protein is used, depends on the nature of the adhesion molecule; for example, ICAM-1 and ICAM-2 both link to actin via ezrin, whereas a closely related cell adhesion molecule with different functions, ICAM-3, does not.

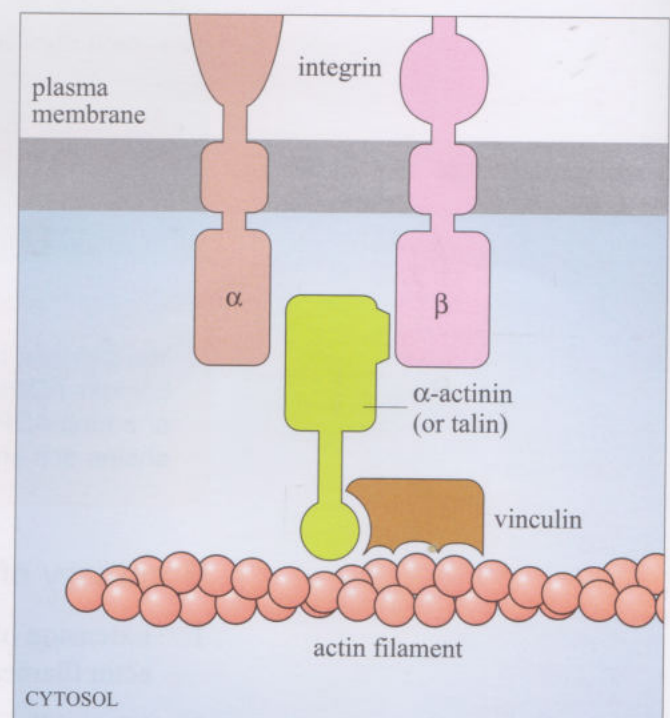
**Figure 16.18**

Inactive ezrin in its folded conformation is activated either by phosphorylation or by binding to phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂), exposing actin-binding and protein-binding domains, which allow it to cross-link molecules such as ICAM-1 to actin filaments.

The activity of the ERM proteins is regulated by both intracellular and extracellular signals. Activation signals can switch the conformation of the anchoring proteins from a folded inactive form to an open active form, which binds actin filaments and promotes cross-linking of adhesion molecules to the actin cytoskeleton (Figure 16.15). We shall look at how various signals can activate the ERM proteins in Section 16.5.

You can see the structure of the N-terminal domain of radixin, complexed with the cytosolic segment of ICAM-2 in ViewerLite™ using the file 1JI9.pdb.

Integrins also bind indirectly to the actin filaments, but use a different set of anchoring proteins, which include α -actinin, vinculin and talin. In addition to stabilizing filamentous actin networks, α -actinin can also cross-link proteins such as integrins to the cytoskeleton (via interactions with the β chain). **Vinculin** is a scaffold protein that binds to actin filaments, and interacts with other anchoring proteins such as α -actinin. You have already encountered talin as a protein that binds to the cytosolic portion of integrins and may induce allosteric changes in the extracellular portion of an integrin to open the MIDAS and promote adhesion (Figure 16.19). Additional scaffold and anchoring molecules have been described, some of which can interact with the α chain of integrins.

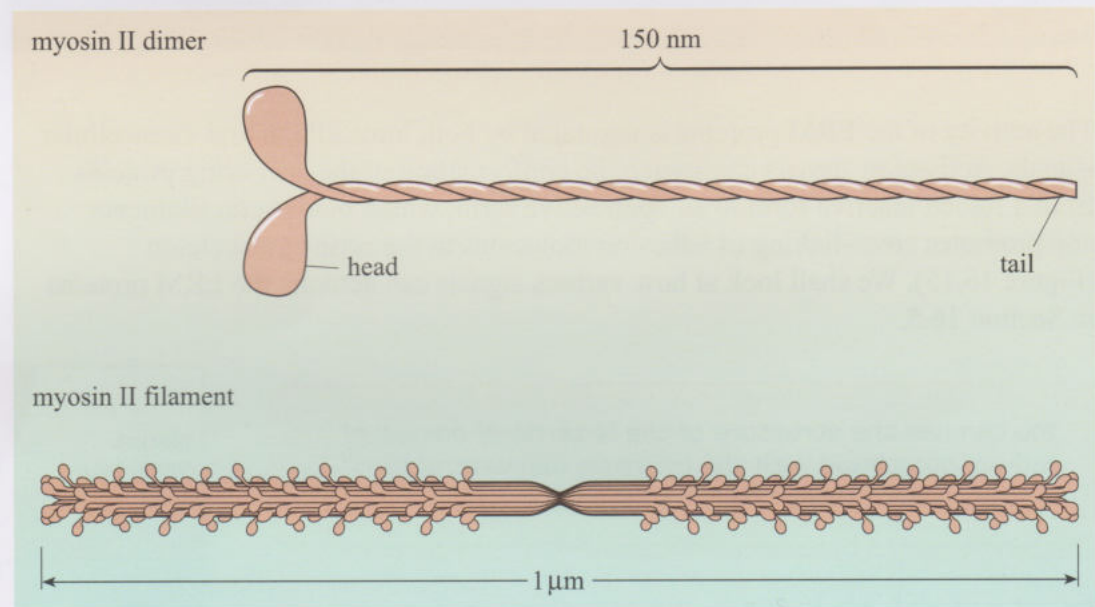
**Figure 16.19** Integrins are attached via their β -chain to actin filaments by molecules such as α -actinin and talin.

- ☐ What is the name of the areas on the cell surface where integrins attach to the extracellular matrix? (*Note:* These are also the areas where bundles of actin filaments forming stress fibres terminate.)
- ☒ These are focal adhesions.

16.4.3 Myosin II contraction pulls the cell forward

For a cell to migrate, it is not sufficient to just extend pseudopodia. The cell body has to follow the leading edge, and this is a function of another cytoskeletal element—**myosin II** (see Box 12.1 and Figure 16.20). Cells of *Dictyostelium* that are deficient in myosin II extend their pseudopodia as normal, but move slowly, because the body of the cell does not keep up. Since myosin II is preferentially located in the uropod (and myosin I in the pseudopod) this leads to the assumption that myosin II contraction is required to pull the trailing edge forwards. Cells deficient in myosin II also tend to form inappropriate lateral pseudopodia, indicating that myosin II helps to pull in the sides of the cell and contributes to lateral tension in the cell. As well as maintaining the overall integrity of the cell shape, myosin is involved in moving organelles: in a migrating cell, mitochondria, the nucleus and other organelles must all be shifted.

Figure 16.20 A single myosin II dimer and a cytoskeletal muscle myosin filament assembled from multiple dimers. The catalytic sites are located in the globular heads.



You can see the structure of the globular region of myosin II using the file Myosin-ADP.msv in ViewerLite™. In this representation, a space-filling model of bound ADP (white) is shown in the catalytic site, and the three different chains are shown in different colours.

Summary of Section 16.4

- 1 Extension of pseudopodia involves polymerization, extension and branching of actin filaments at the leading edge of the cell.
- 2 Many adhesion molecules, including integrins and Ig superfamily CAMs interact with actin filaments via anchoring proteins. Engagement of adhesion molecules with the cytoskeleton depends on the arrangement of the cell and whether the cell has been activated by extracellular signals.

- 3 The ERM family proteins—ezrin, radixin and moesin—act as cytoskeletal anchoring proteins for several transmembrane proteins including Ig superfamily CAMs. Following activation, these proteins switch to an open conformation, which cross-links the adhesion molecule to actin filaments.
- 4 α -Actinin, vinculin and talin are involved in cross-linking integrins to filaments.
- 5 Myosin II interacts with filaments, to maintain the shape of the cell, for moving the cell forward and to move organelles.

16.5 Chemotactic molecules and their receptors

The sections above have described the basic mechanisms of migration and the molecules involved in adhesion, but the key to cell movement is the coordination of attachment, movement and detachment in response to an external stimulus. Complete explanations of this process are not yet available, but a considerable amount is known about the signalling mechanisms involved in chemotaxis.

Most studies of chemotaxis involve cell polarization and movement towards the chemotactic stimulus. In *Dictyostelium*, for example, single cells move towards each other by releasing chemotactic molecules, which attract other cells. For leukocytes, however, cells in the tissues release chemokines, which attract neutrophils and other leukocytes from the blood, causing them to cross the endothelium and then to migrate to a site of inflammation. Leukocytes that have entered an area of inflammation can also release chemokines, which cause the accumulation of further cells migrating from blood. In contrast to the numerous examples of cell movement towards a stimulus, far fewer studies have addressed the problem of how cells stop moving or move away from stimuli, even though this subject is equally important.

16.5.1 Chemotactic molecules

Bacterial chemotaxis towards nutrients such as amino acids or sugars is directed by activation of specific enzyme-associated receptors involving histidine kinases. In other organisms, many chemotactic responses are mediated by 7-helix transmembrane G protein-coupled receptors (GPCR) on the cell surface. For example, the chemotactic response to cAMP, and aggregation of individual *Dictyostelium* amoebae into multicellular aggregates, occurs via activation of a GPCR. Despite basic similarities in the intracellular signalling systems in multicellular animals, there is a wide range of types of extracellular signalling molecule and of mechanisms of GPCR activation (Section 13.2.2). Table 16.3 gives examples of some of the classes of chemotactic molecule that act on vertebrate neutrophils and macrophages—phagocytes that are involved in defence against infection and clearing debris.

Table 16.3 Some chemotactic molecules acting on leukocytes in vertebrates.

Molecule	Type	Source
fMet–Leu–Phe (fMLP)	formylmethionine peptides	bacterial protein synthesis
interleukin-8 (IL-8)	chemokines (small proteins)	endothelium, tissue cells
C5a	peptide fragment of complement system molecule C5	protein found in blood
leukotriene B4 (LTB4)	eicosanoid	mast cells, macrophages

- ☐ What advantage is there in having so many different types of chemotactic molecule?
- ☒ The great diversity is related to the wide range of stimuli that may cause tissue damage and inflammation, and require the presence of phagocytes.

We shall now look in detail at the examples in Table 16.3.

Bacteria initiate their mRNA translation using formylmethionine (fMet), whereas eukaryotic cells initiate synthesis with methionine (Section 12.4.1). Consequently, the presence of fMet peptides is a marker of bacterial infection, and attracts neutrophils and macrophages to phagocytose the bacteria.

C5a is a polypeptide cleaved from a larger complement system molecule C5, by an enzyme that is expressed following a series of activation pathways initiated by antigen–antibody complexes or foreign cell surfaces. Hence, C5a is a marker of an ongoing defensive immune reaction.

Leukotriene B4 (LTB4) is a product of arachidonic acid metabolism. The class of molecules generated from arachidonic acid are lipids called ‘eicosanoids’, and many are mediators of inflammation. They are produced by a variety of cells in damaged tissues, including *mast cells*, in response to a variety of signals. Consequently, LTB4 is a marker of tissue damage.

Interleukin-8 (IL-8) is just one member of the largest class of vertebrate extracellular signalling molecules, the chemokines, and you will be investigating this protein in some detail.

Chemokines are a very large family of extracellular signalling proteins: at least 70 members have been identified in mammals. Most of them are diffusible molecules with M_r approximately 10^4 , but some members are produced as plasma membrane-bound forms. Originally they were identified for their roles in attracting leukocytes to sites of inflammation, where they induce triggering of leukocytes following initial rolling on endothelium (Figure 16.2). However, it has become increasingly obvious that they are also involved in many developmental processes, as well as the localization of specific subsets of leukocytes to the appropriate areas of lymphoid tissues. Chemokines are subdivided into different families, according to their structure. For example, some chemokines have a characteristic pair of cysteine residues, separated by one other amino acid—a CXC motif. Any of the chemokines belonging to this family are called CXC ligands, for example CXCL1, CXCL2, etc. In fact, most of the chemokines were given other names before they

Mast cells are located in tissues near blood vessels and contain large numbers of granules with inflammatory mediators.

were placed into this unified system. So the chemokine interleukin-8 (IL-8) mentioned above, is now designated 'CXCL8'. The structure of another chemokine, in this case belonging to the CC family (the two cysteine residues are not separated by another amino acid), is shown in (Figure 16.21).

We are going to look in some detail at the structure and function of IL-8 as a model for the actions of other chemokines. IL-8 is involved in the migration of leukocytes out of blood vessels as well as promoting chemotaxis within tissues. Endothelial cells secrete IL-8 at low levels constitutively, and synthesis is greatly increased in response to inflammatory signals from the tissues. IL-8 is stored in Weibel Palade bodies (a storage vesicle found in endothelium; see Figure 16.12), and is released to the apical surface (blood side) of the endothelium. Like many other chemokines, IL-8 is able to bind to glycosaminoglycans associated with endothelial cell surface molecules, so that it is held at the cell surface in a position where it can signal to tethered leukocytes (see the triggering stage of Figure 16.2). As a leukocyte travels with the blood flowing through a small vein, it can potentially receive a signal from the chemokines present on the endothelial surface, provided it expresses the appropriate chemokine receptors. There are two receptors for IL-8, CXCR1 and CXCR2. (Note the designation for chemokine receptors: CXCR means a receptor for a CXC or a family chemokine, and in this case it is receptor numbers 1 and 2—1R1 and R2.)



Figure 16.21 A molecular model of the CC chemokine CCL20 (also known as MIP-3 α or macrophage inflammatory protein-3 α). These small molecules frequently crystallize as dimers with an area of β pleated sheet and two α helices. There is evidence that dimers are the more active physiological form.

Molecular modelling 9

You should now go to the Study Skills file: *Molecular modelling 9*, to carry out a study of the chemokine interleukin-8 (IL-8). The aim of this investigation is to determine which regions of IL-8 are involved in binding to glycosaminoglycans (on the endothelial cell surface or in the ECM), and which regions are involved in binding to the chemokine receptors CXCR1 and CXCR2 (on the leukocyte surface). Most chemokines that have been investigated are structurally similar to IL-8, and many have a glycosaminoglycan-binding capacity, which allows them to attach to cells or to the extracellular matrix.

Molecular modelling 9 should have given you some insight into how IL-8 is bound at the surface of endothelium and how it binds to its receptors on leukocytes. Tethered leukocytes are activated by chemokines in proportion to the numbers of receptors that are activated. (The intracellular signals involved in activation are described in Section 16.6.) Activation causes leukocytes to change the binding affinity of their integrins, so that they become firmly attached to adhesion molecules, such as ICAM-1, expressed on the endothelium (see the adhesion stage of Figure 16.2). The longer a leukocyte remains tethered to the endothelium, the greater the signal that it can receive, so leukocytes effectively integrate the chemokine signals over time. Studies *in vitro* suggest that if 100–1000 molecules of IL-8 bind to its receptors on neutrophils (CXCR1 and CXCR2), this leads to the conversion

of roughly 20 000 integrin molecules to high-affinity forms – sufficient for the neutrophil to attach firmly to the endothelium. Once cells have attached, they crawl through the endothelium, and then through the basal lamina, before migrating into the inflamed tissues.

A description of G protein-coupled receptors is given in S204, Book 3, Chapter 6, and additional information is in Section 13.3 of this course. You may find it helpful to review this information before the next section.

16.5.2 G protein-coupled receptors for chemokines

The different receptors that recognize chemotactic molecules vary in their degree of specificity. For example, the receptor for C5a (C5aR) binds only to C5a, whereas the two receptors for IL-8 identified above also recognize other chemokines. When a receptor binds to several different ligands, or a ligand binds to several different receptors, it is referred to as ‘promiscuous binding’, for which chemokines and their receptors are notable. The majority of chemokines bind to more than one receptor, and most receptors bind several chemokines. It is therefore difficult to predict exactly how a cell will respond in a physiological situation where it may be subject to a variety of chemotactic signals acting on different receptors. (Note that the chemokine receptors that bind to the CXC chemokines are designated CXCR1–CXCR6, whereas those that bind to the CC chemokines are CCR1–CCR11). Even though cells are polarized and are capable of differentiating very small differences in the concentrations of chemotactic molecules, the distribution of chemokine receptors in unstimulated cells is almost uniform around the cell surface.

16.5.3 Signalling polarization

To understand the following discussion, you may find it helpful to review Section 13.3 and Figure 13.30, which describe the phosphorylation of phosphatidylinositol and its derivatives by kinases.

Activation of PI 3-kinase (Book 3 Section 13.3.2, p. 110) appears to be critical in the intracellular signalling pathways activated by the GPCRs that control chemotaxis. This is true in *Dictyostelium*, fibroblasts and leukocytes. In mammalian cells, there are several classes of PI 3-kinase, but here we are concerned with PI 3-kinase γ , which is activated by the $\beta\gamma$ complex of heterotrimeric G proteins (in contrast with other PI 3-kinases, which are activated by RTKs, as explained in Chapter 13).

- ☐ When antibodies that bind PI 3-kinase γ were microinjected into neutrophils, they were prevented from responding to a chemotactic stimulus, whereas antibodies against the related kinase PI 3-kinase α had no such effect. How do you interpret this experiment? (The technique of microinjection is explained in Box 13.3.)
- ☒ Microinjected antibodies inhibit the activity of PI 3-kinase γ or PI 3-kinase α by binding to them within the cell. Since the cells no longer respond to a chemotactic stimulus only when antibodies against PI 3-kinase γ are introduced into cells, it implies that the γ isoform of PI 3-kinase, but not the α isoform, is a component of the signalling pathway mediating the chemotactic response of neutrophils.

In accordance with this theory, leukocytes from animals genetically deficient in PI 3-kinase γ show defective polarization and chemotaxis. Active PI 3-kinase γ phosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) to produce phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃), which is able to recruit a number of proteins with **pleckstrin homology (PH)** domains to the cytosolic side of the plasma membrane (Figure 16.22). Pleckstrin homology domains bind to PI(3,4,5)P₃ and are found in a variety of proteins that are recruited to the plasma membrane (Figure 13.31). These include protein kinase B (PKB) in leukocytes, and *Dictyostelium*, and two other proteins, PhdA (PH domain-containing protein A) and CRAC (cytosolic regulator of adenylyl cyclase), which are found only in *Dictyostelium*. When cells are stimulated globally with a chemotactic molecule, these PH domain proteins redistribute uniformly throughout the cell membrane, but if the stimulus is applied to just one side of the cell, PH domain-containing cytosolic proteins dock onto the plasma membrane only on the edge where the chemotactic stimulus was applied. PH domain proteins are all important in chemotaxis, but perform different functions. For example, PhdA is required for proper assembly of F-actin, whereas the precise function(s) of PKB, although essential for chemotaxis, is unclear. Once it has been recruited to the plasma membrane, PKB itself becomes activated by another kinase, PDK1, which also has a PH domain (hence it is also recruited to the plasma membrane). The overall effect of activation with chemotactic molecules, therefore, is to cause a number of proteins that are required for chemotaxis to associate with the leading edge of the plasma membrane.

Protein kinase B was independently called Akt, and now is often referred to as PKB/Akt (Chapters 13 and 14).

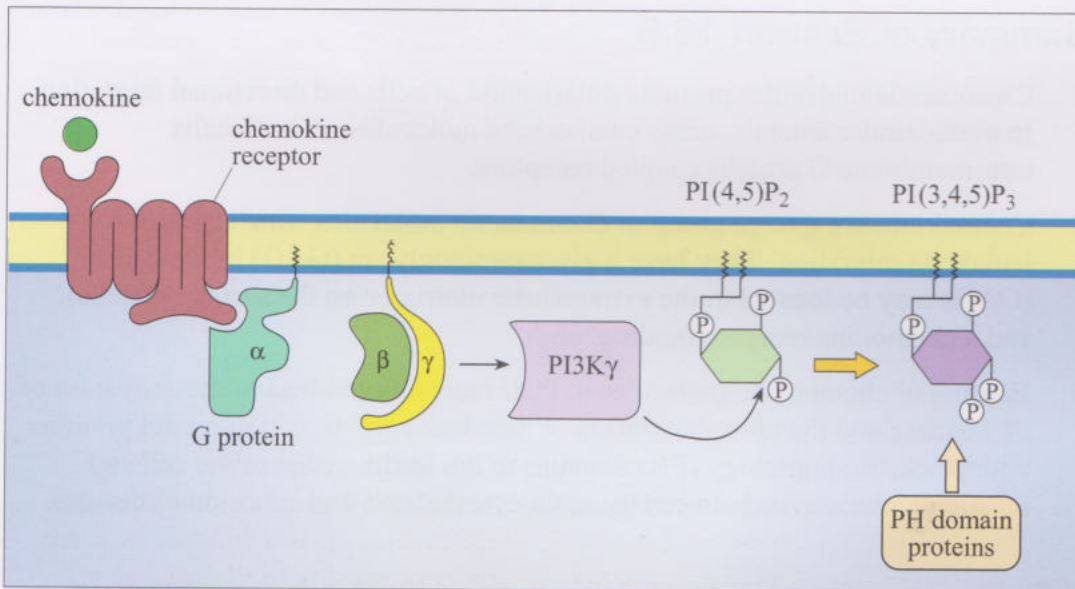


Figure 16.22 Chemokines bind to chemokine receptors, which are 7-helix transmembrane G protein-coupled receptors. Binding causes the dissociation of the trimeric G protein into activated α and $\beta\gamma$ subunits. The $\beta\gamma$ complex binds and activates PI 3-kinase γ (denoted as PI3K γ in the figure) which causes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), producing phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃). This second messenger binds to pleckstrin homology (PH) domains present on a number of proteins such as protein kinase B (PKB). Thus, signalling by chemotactic agents recruits these proteins to the leading edge of the cell.

- ☐ Predict what would happen if the PH domain of PKB was mutated so that binding to PI(3,4,5)P₃ could no longer occur.
- ☒ The mutant PKB could not bind to PI(3,4,5)P₃ (its docking site on the plasma membrane), so it could not be activated by PDK1, as recruitment to the plasma membrane has been abolished. As a result, chemotactic responses would be impaired in cells with such mutations.

You can see how CRAC (Figure 16.24) is mobilized to the leading edge of the plasma membrane of migrating *Dictyostelium* in the movies 'Chemotaxis of *Dictyostelium* I and II' located in the Cell migration folder of the *Movie Gallery*.

Also important in cell polarization are the guanine nucleotide exchange factors (GEFs), which contain PH domains and associate with PI(3,4,5)P₃ at the plasma membrane. These proteins regulate the activation of a family of small GTPases, including those belonging to the Rho family, described below, which are central to the reorganization of the cytoskeleton in moving cells. In addition, PH domain-containing proteins modulate adhesion molecule affinity and clustering, and cause adhesion molecules to bind to the filaments.

Although the gradient of chemotactic molecules between the pseudopod and the uropod is quite small, the signalling process described above, involving PI 3-kinase γ appears to selectively amplify the signals, so promoting cell polarization. The speed with which cells respond to chemotactic molecules is impressively swift: within seconds of application, PKB and other PH domain-containing proteins start to localize to the leading edge of the cell. PI 3-kinase γ appears to be universally involved in chemotaxis of a very wide variety of cell types, and many different chemotactic responses are inhibited by agents that interfere with PI3-kinases.

The link between PI 3-kinase γ and the other signalling events involved in cell migration are only poorly understood. The proposed intracellular signalling pathways involve a number of steps that result in the polymerization of F-actin, and may be the direct result of, for example, activation of PhdA (in *Dictyostelium*), or they may be indirect, involving the Rho family of small GTPases, as described in Section 16.6.

Summary of Section 16.5

- 1 Chemotactic molecules promote polarization of cells and directional migration. In multicellular animals, many chemotactic molecules act on 7-helix transmembrane G protein-coupled receptors.
- 2 Chemokines are a large group of chemotactic molecules which control leukocyte migration. They have a glycosaminoglycan (GAG) binding site (GAGs may be located in the extracellular matrix or on the surface of cells), and a chemokine receptor binding site.
- 3 Binding of chemotactic molecules to their receptors can lead to the activation of PI 3-kinase and the phosphorylation of membrane lipids, which recruit proteins with pleckstrin homology (PH) domains to the leading edge of the cell and modulate interactions between the actin cytoskeleton and adhesion molecules.

16.6 Control of cell adhesion and chemotaxis

16.6.1 Rho-family GTPases control cell polarization

Cell adhesion and cell movement require integration of the intracellular processes controlling the attachment of adhesion molecules (to the ECM or to other cells), and those that control the organization of the cytoskeleton. Some of the steps in this process, and the key molecular players, are known, but a complete description is not yet available. One important group of proteins that affect the organization of the cytoskeleton are the **Rho-family GTPases**. This is a subgroup of the much larger family of small GTPases, the Ras superfamily. The principal members of the Rho family are **RhoA**, **Rac** and **Cdc42**. Activation of these small G proteins (by GEFs

Do not confuse Rho-family GTPases with the transcription termination factor, also called Rho.

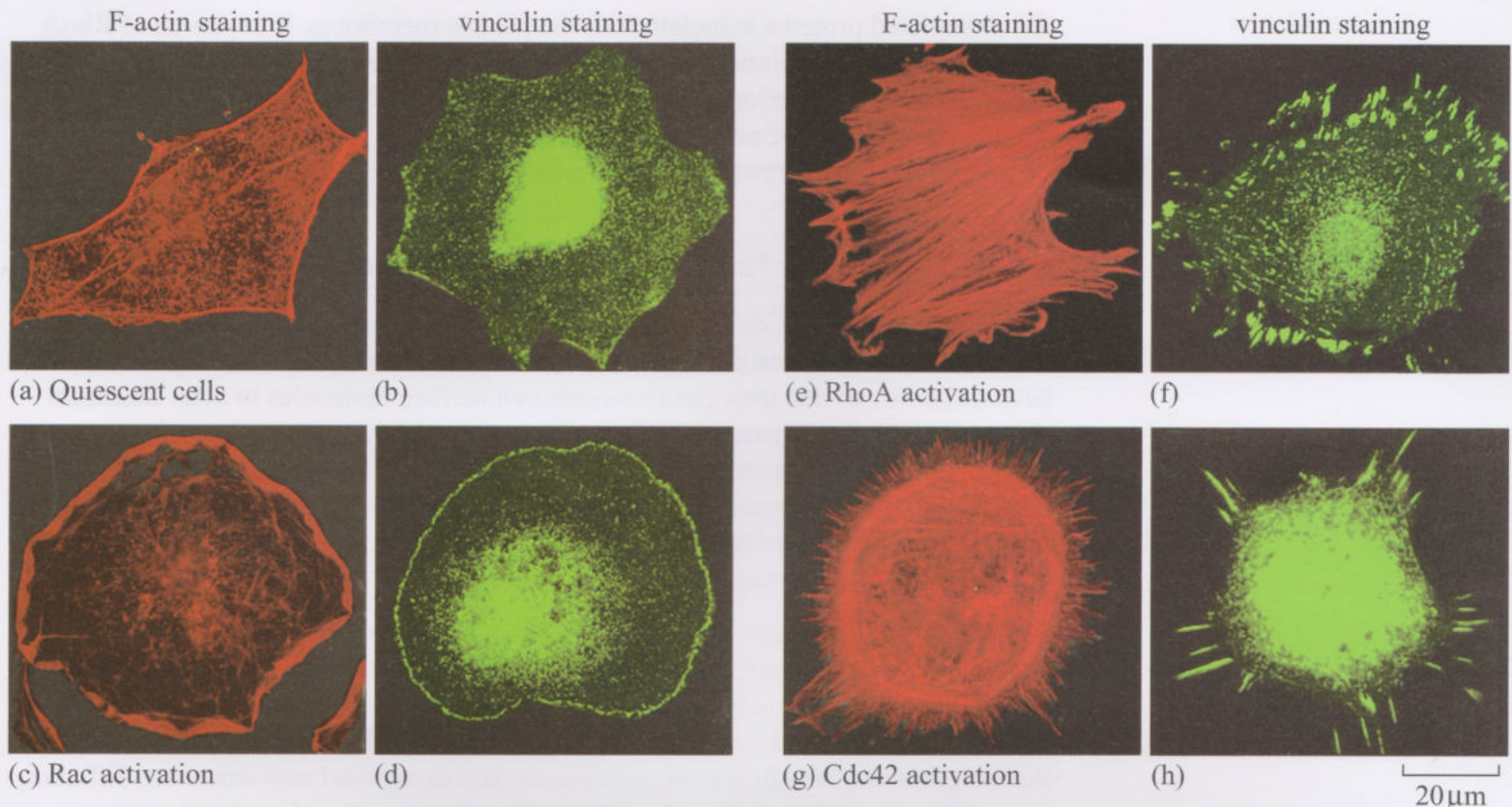


Figure 16.23 The role of Rho-family GTPases in controlling the organization of the cytoskeleton. Constitutively active RhoA, Rac and Cdc42 were injected into single fibroblasts, and the cells stained with fluorescent probes to detect F-actin (a, c, e and g) or vinculin (b, d, f and h). (a, b) Normal quiescent fibroblasts have a finely distributed network of F-actin with filaments concentrated around the margin of the cell; vinculin is generally distributed across the cells, but concentrated around the cell body. (e, f) Activation of RhoA causes the cells to polarize, inducing stress fibres and causing vinculin to localize to focal adhesions. (c, d) Activation of Rac causes both F-actin and vinculin to distribute around the cell periphery, forming a uniformly distributed lamellipodium. (g, h) Activation of Cdc42 induces filopodia formation and translocation of vinculin to the filopodia.

recruited to the plasma membrane) leads to global reorganization of the cytoskeleton. This is shown for fibroblasts in Figure 16.23, but these molecules are equally important in controlling leukocyte migration.

The control of Ras activation is illustrated in S204, Book 3, Figure 6.42, and a simplified scheme of how the activity of this family of proteins is regulated is shown in Figure 13.8 of this course.

The effects of the three GTPases are quite distinct, as can be seen in Figure 16.23. Activation of RhoA causes the cells to polarize, inducing stress fibres and focal adhesions, whereas activation of Rac induces the formation of a uniformly distributed lamellipodium. When Cdc42 is activated, filopodia formation is induced. It is thought that normally just one of the GTPases might become active, which changes the overall shape and function of the cell—that is, spreading or directional movement.

- ☐ RhoA may be prenylated, and inhibitors of RhoA prenylation also inhibit the formation of focal adhesions. What is the significance of this?

- Prenylated proteins associate with the plasma membrane. Prenylation of RhoA promotes its recruitment to the plasma membrane. Since preventing prenylation inhibits focal adhesion formation, translocation of RhoA from the cytosol to the membrane must be necessary for its activation, and is also involved in controlling the formation of focal adhesions.

16.6.2 The Rho-family GTPases also modulate adhesion

The effects of RhoA, Cdc42 and Rac on the cytoskeleton require proteins of the ERM family. It has been proposed that RhoA either directly or indirectly activates ERM proteins, so that they can cross-link cell surface molecules to actin filaments (Figure 16.18). Furthermore, the interaction of ICAM-1 on the surface of endothelial cells with extracellular ligands, leads to activation of RhoA and reorganization of the cytoskeleton, indicating that RhoA both affects adhesion and may itself be modulated by adhesion. However, the precise signalling links along this pathway are uncertain.

The Rho-family GTPases are also involved in the interactions of integrins with the actin network at focal adhesions: cytoskeleton-associated proteins such as vinculin and talin that bind to the $\beta 1$ chain of integrins and the ARP complex are all differentially regulated by RhoA, Cdc42 and Rac via other signalling proteins. Since the integrins involved in spreading/adhesion and directional movement are different, one might expect that each of the GTPases would interact with and activate one specific group of integrins, although the details of this are not known. You should also recall that the integrins are two-way signalling molecules, whose activity is potentially modulated by the nature of the extracellular matrix or of neighbouring cells. Rho-family GTPases may also therefore be involved in relaying signals from the cell surface adhesion molecules to other intracellular signalling pathways and the cytoskeleton.

From the descriptions of adhesion, migration and chemotaxis, one can see that we have islands of understanding in a sea of uncertainty. Often we know that there must be some link between components, such as between RhoA activation, focal adhesion formation, integrin clustering, and F-actin reorganization, but we do not know exactly how the links are made. Figure 16.24 highlights some of the known connections between the signalling systems and cell movement, based on studies in *Dictyostelium*.

16.6.3 Inhibition and control of chemotaxis

Once a cell has moved up a chemotactic gradient towards its source, we might expect it to stop there, since there is no longer a gradient. However, cell movement often involves successive stages. For example, during development, neurons move progressively from one area of the embryo to another along well-defined but complex pathways (Chapter 17). Similarly, in inflammation, cells move progressively from one area within the body to another, responding to successive chemotactic stimuli. For example, neutrophils first migrate from blood across endothelium, and then move through the tissues towards a source of infection or tissue damage. In these cases, the cells become insensitive to one chemotactic stimulus but retain sensitivity to another, in a process that may involve interactions between different components of the signalling pathways triggered by different GPCRs or the loss of receptors to chemotaxis from the cell surface by endocytosis.

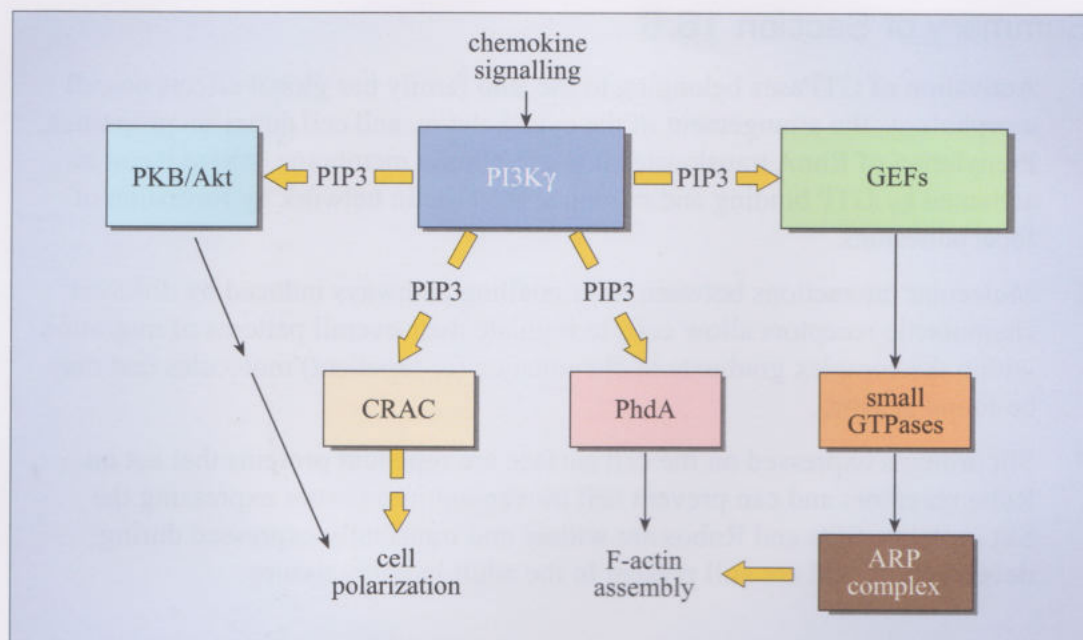


Figure 16.24 Signalling systems that reorganize the cytoskeleton. Activated PI 3-kinase γ (denoted as PI3K γ in the figure) causes local generation of PI(3,4,5) P_3 (PIP3), which recruits a number of proteins to the cell membrane, including protein kinase B (PKB), cytosolic regulator of adenylyl cyclase (CRAC), PH domain-containing protein A (PhdA) and guanine nucleotide exchange factors (GEFs). These mediators either directly or indirectly cause cell polarization and assembly of F-actin networks at the plasma membrane.

In addition to these interactions, there are specific mechanisms that inhibit chemotaxis. The best-studied system involves a group of receptors which were first identified in migrating neurons of developing *Drosophila*. In flies with mutated forms of these receptors, the directional movement of neurons in development was defective, as they would migrate in inappropriate 'roundabout' pathways. Hence, the receptors were called **Robo receptors** (Robo receptors are single-pass transmembrane recruiter receptors that interact with Rho family GTPases on activation). The ligands for these receptors are members of the **Slit** family of proteins (the name of the gene product of the *Drosophila slit* locus), which may be bound to the extracellular side of the plasma membrane or may be released as diffusible molecules. In mammals, there are three types of Robo receptors and three types of Slit, which display a degree of promiscuous binding. When a cell with a Robo receptor contacts a cell expressing an appropriate Slit molecule, migration is inhibited, by a mechanism that interferes with signalling from G protein-coupled chemotactic receptors. In some cases, dimerization of Robo receptors prevents the activation of Rho-family GTPases induced by chemotactic molecules. Developing neurons express combinations of different Robos, thereby inhibiting the movement of certain neurons into specific areas of the developing nervous system. The expression of Robos on neurons, in association with the action of chemotactic molecules, channels them into specific migratory pathways during development.

Initially it was thought that the Slit/Robo system was confined to the nervous system, and that its only function was to guide migrating neurons during development. However, these molecules are also expressed in adult animals, and the receptors have also been found on populations of leukocytes. It now appears that Slits and Robos represent a more general system for inhibiting and controlling the migration of many different cell types including neurons, leukocytes and endothelial cells.

Summary of Section 16.6

- 1 Activation of GTPases belonging to the Rho family has global effects on cell morphology, the arrangement of the cytoskeleton, and cell adhesion properties. Prenylation of RhoA translocates it to the plasma membrane, where it can be activated by GTP binding and modulate the F-actin network by formation of focal adhesions.
- 2 Molecular interactions between the signalling pathways induced by different chemotactic receptors allow cells to regulate their overall patterns of migration within the complex gradients of chemotactic (or repellent) molecules that may be found *in vivo*.
- 3 Slit proteins expressed on the cell surface are repellent proteins that act on Robo receptors and can prevent cell movement into tissues expressing the Slit proteins. Slits and Robos are widely and transiently expressed during development, and are still present in the adult in many tissues.

16.7 Disintegrins and metalloproteases

Once a leukocyte has crossed endothelial cells and entered the tissue, it has to move through a dense network of cells and extracellular matrix components, of which the first is the basal lamina of the endothelium (the diapedesis stage shown in Figure 16.2). Leukocytes move through tissues by releasing enzymes that cleave the components of the extracellular matrix. The enzymes responsible for these functions belong to two related families, the **matrix metalloproteases (MMPs)** and the **metalloprotease disintegrins** ('a disintegrin and metalloprotease', **ADAMs**). Both sets of molecules have a protease domain, which depends on zinc ions for its catalytic activity and which degrades extracellular matrix components. However, MMPs are secreted molecules, whereas ADAMs are transmembrane proteins that contain, in addition to the metalloprotease domain, a 'disintegrin' domain affecting cell adhesion. There are more than 10 proteins in each of the families, with subtly different substrate specificity.

Migrating leukocytes release MMPs from intracellular stores. For example, as leukocytes migrate across endothelium, metalloproteases are secreted by the leukocyte. The released metalloproteases digest the basal lamina of the endothelium, and allow the cell to access the tissues. The activity of MMPs and ADAMs is inhibited by another family of enzymes, the **tissue inhibitors of metalloproteases (TIMPs)**. A balance is maintained between the activity of the metalloproteases and their inhibitors, so that the release of metalloproteases by migrating cells does not lead to uncontrolled digestion of tissues.

In addition to their role in cell movement and inflammation, metalloproteases are involved in tissue remodelling following damage, and some MMPs cause the release of growth factors bound to the extracellular matrix. As a result, growth factors are released, which promote regeneration as tissue is remodelled.

Metalloproteases have also come into prominence because of their role in tumour development. Although this chapter has concentrated exclusively on two patterns of cell movement (*Dictyostelium* aggregation and leukocyte migration), many of the processes described here are equally relevant to other situations, such as tumour development. Invasive tumours lose their normal adhesion properties, reorganize

their cytoskeleton, and may become mobile and invasive. A key element of invasiveness is the ability to secrete metalloproteases, which allows the cells to move away from the initial site of tumour development. Ultimately, invasive cells may break in to blood vessels or lymphatics and spread through the body, a process called 'metastasis', which is described in more detail in Section 19.2.8.

Summary of Section 16.7

- 1 Matrix metalloproteases (MMPs) are secreted by migrating cells, which allows them to move through tissues by cleaving molecules of the extracellular matrix.
- 2 Metalloprotease disintegrins (ADAMs) interfere with intercellular adhesion, and also can facilitate leukocyte motility.
- 3 MMPs and ADAMs are inhibited by tissue inhibitors of metalloproteases (TIMPs). The balance of the enzymes and inhibitors is important in controlling cell motility and tissue remodelling.

Learning outcomes for Chapter 16

After completing this chapter, you should be able to:

- 16.1 Define and use all the words given in **bold** in the text.
- 16.2 Outline the role of cell migration in the positioning of cell types within the organism during development or in adult life.
- 16.3 Describe the molecular mechanisms by which cells adhere to the extracellular matrix or to other cells, and the ways in which adhesion can be controlled at a molecular level.
- 16.4 Give examples of a number of adhesion molecules, and the ways in which they interact with their extracellular ligands and engage with the cytoskeleton.
- 16.5 Describe, with examples, how directional migration of cells occurs in response to chemotactic stimuli, and outline some of the molecular processes underlying chemotaxis.
- 16.6 Understand why particular adhesion molecules have evolved for different types of cell adhesion and movement.

Questions for Chapter 16

Question 16.1

Name five types of cell that have the potential to move through tissues of an adult mammal. What functions does cell migration serve in an adult mammal?

Question 16.2

The molecule E-cadherin is normally expressed on epithelial cells; fibroblasts do not express it. If fibroblasts were transfected with a plasmid containing the gene for E-cadherin so that E-cadherin was constitutively expressed in the cells, what effect would you expect it to have on the adhesion of fibroblasts when grown *in vitro*? What effect on cell–cell adhesion would you expect to see if fibroblasts were transfected with a plasmid containing the gene for ICAM-1. (*Hint* Consider the extracellular ligands that E-cadherin and ICAM-1 bind to.)

Question 16.3

For the following proteins, indicate the class of molecule they are (e.g. anchoring protein, receptor, etc.), and state their function in cell adhesion and/or migration: C5a, Cdc42, CXCR1, ezrin, PI 3-kinase, Robo-1, vinculin.

Reference

Svitkina, T. M. and Borisy, G. G. (1999) Arp2/3 Complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia, *Journal of Cell Biology*, **145** (5), pp. 1009–1026.

Further sources

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002) *Molecular Biology of the Cell* (4th edn), Garland Science, New York.

Chung, C. Y., Funamoto, S. and Firtel, R. A. (2001), Signalling pathways controlling cell polarity and chemotaxis, *Trends in Biochemical Sciences*, **26**, pp. 557–566.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton, *Science*, **279**, pp. 509–514.

Hynes, R. O. (2002), Integrins: bidirectional, allosteric signalling machines, *Cell*, **110**, pp. 673–687.

Weijer, C. J (2003) Visualizing signals in moving cells, *Science*, **300**, pp. 96–100.

17 DIFFERENTIATION OF ANIMAL CELLS

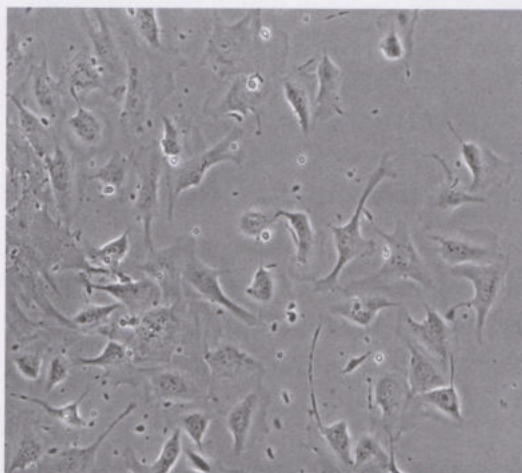
17.1 Introduction: what is differentiation?

In this chapter, we shall build on material that was covered in S204, Book 3 *The Core of Life*, Vol. II, Section 10.2, which provides background for the topics covered here.

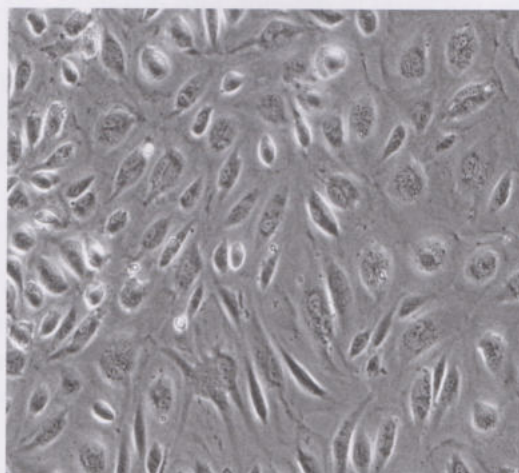
Most multicellular organisms are composed of a variety of different cell types, specialized to perform specific functions. The process by which a cell becomes specialized is known as **differentiation**. This term, however, encompasses a number of different molecular and cellular processes, many of which you have already studied in this course.

- ☐ By now, you should have an understanding of what distinguishes different specialized cells from each other. List some of the ways in which cells differ, at the cellular and the molecular levels.
- ☒ You might have suggested several properties, e.g. size, shape, position, interrelationships, function, protein content, mRNA content, transcription factors, cell surface molecules, etc.

At the cellular level, different types of cells can differ in size, have specialized shapes and specific relationships with other cells (Figure 17.1). These cellular properties are determined at the molecular level, by the proteins that the cells express. For example, epithelial cells are closely apposed and form sheets, due to their expression of specialized proteins (e.g. cadherins and other junctional proteins; Chapters 6 and 16). The different types of protein that a cell expresses ultimately determine the fate of the cell, and in turn depend upon the pattern of gene expression by the cell.



(a)



(b)

100µm

Figure 17.1

Phase contrast micrographs of two cell types in primary cultures prepared from an adult human brain specimen. (a) Astrocytes (a type of glial cell), which have an irregular shape and do not form close contacts. (b) Endothelial cells, which are regular in shape and, as they make close contacts with each other, tend to form sheets. (Courtesy of Dr I. Romero.)

It is important to realize that differentiation involves a sequence of molecular events, and also that the first steps along the pathway to differentiation into a particular cell type may not commit the cell to that fate.

A cell is said to be **specified**, if it has received signals that result in it (or its progeny) later assuming a certain phenotype, but this does not mean that, if it later receives additional or other signals, it may not follow a *different* developmental pathway.

A cell is said to be **committed** or **determined** once the signals that it has received result in changes that are set, and its fate is decided. Under normal circumstances, although committed cells are not fully differentiated, their fate cannot be changed. Note however that, in some circumstances, the properties of even fully differentiated cells can be changed. One important example is in the transformation of cells that results in tumour development (Chapter 19). We return to this topic in Section 17.4.

Differentiation of cells takes place within both developing and mature tissues. You may recall (from S204) that **development** includes all of the events that occur during the formation of a mature organism from a zygote (fertilized egg). Development involves not only cellular differentiation, but also the formation of tissues – groups of cells that together perform a particular function or functions, but that may sometimes contain different cell types. For example, skeletal muscle contains connective tissue cells and cells of the vascular system and nerve fibres, as well as muscle cells (called fibres).

- ☐ In addition to differentiation, which other cellular processes are involved in development?
- ☒ Cell division, growth, migration and death (Chapters 8, 14, 15 and 16).

The successful development of an organism depends upon tissue development, which involves appropriate cellular differentiation, the correct balance of cell proliferation and cell death, and also accurate cell migration. In mature, fully developed organisms, tissue maintenance also involves these processes; cells lost by natural turnover, such as red blood cells and the epithelial cells that line the gut and the surface of the skin, are replaced by cells that originate from a pool of undifferentiated cells known as adult **stem cells**. Stem cells are undifferentiated cells that divide to give rise both to more stem cells and also to cells that go on to differentiate into mature functional cells. They are **multipotent**; that is, they have the potential to develop into different cell types of the tissue in which they originate. Evidence indicates that stem cells exist in most tissues of adult animals. In some cases, such as the lymphoid system (Figure 15.2) and epithelial tissues, where there is a rapid turnover of cells, these stem cells continually produce progeny that form differentiated cells. In other cases, such as the nervous system, the normal roles of adult stem cells are unknown, and are the subject of much research. We return to stem cells in Sections 17.3.1 and 17.4.2.

Differentiation depends upon the proteins that a cell expresses, which is determined by differential gene expression and post-transcriptional modification (Chapters 10 and 11). During differentiation, expression of some genes is upregulated, while other genes may be inactivated, or repressed.

- ☐ What is another term used to describe repression of a gene?

■ Gene silencing (Section 10.5).

Gene expression is in turn influenced by gene regulatory proteins (Chapter 10) and by epigenetic factors (Chapters 9 and 10).

□ What are the epigenetic factors that influence gene expression?

■ Histone modifications and DNA methylation, both of which affect chromatin structure and compaction (Section 10.5).

But what determines which epigenetic factors and gene regulatory proteins act on a cell? Research on the development of different cell types in a range of organisms has shown that there are a relatively small number of common mechanisms that drive differential gene expression. We examine these mechanisms in the next section.

Summary of Section 17.1

- 1 Differentiation is the process by which cells become specialized; it involves a sequence of molecular events that result in differential gene expression, which, in turn, determines the proteins that a cell will express.
- 2 Differentiation takes place both during development and in mature tissues.

17.2 Common mechanisms and molecules underlie differentiation

In this chapter, we focus on examples of differentiation in model organisms from the animal kingdom, particularly the vertebrates; we use the clawed frog, *Xenopus laevis*, as our main example, but we also refer to the chicken, *Gallus gallus*, the mouse, *Mus musculus*, and invertebrates including *Caenorhabditis elegans* and *Drosophila melanogaster*. Although all these animals are very different, and they have different life cycles, the molecular basis of their embryonic development is remarkably similar.

17.2.1 Overview of animal development

As we shall look in some detail at the molecular events that result in cellular differentiation during development, we begin by giving an overview of the main stages in this fascinating process. It is important to remember that these stages are not isolated events, but merge into each other; development is a continuous process. Remember also that massive cell proliferation takes place during development, and changes in the properties of cells occur along with their division and also their movement. The three-dimensional movements that occur during development are complex, and although they follow the same general pattern in most animals, the precise cellular arrangements, as well as the overall shape and appearance of early embryos, differ considerably, even among vertebrates such as amphibians, birds and mammals. For simplicity, to illustrate the general processes of development we have therefore chosen to concentrate mainly on just one example in this summary, the frog *Xenopus laevis*, as shown in Figure 17.2.

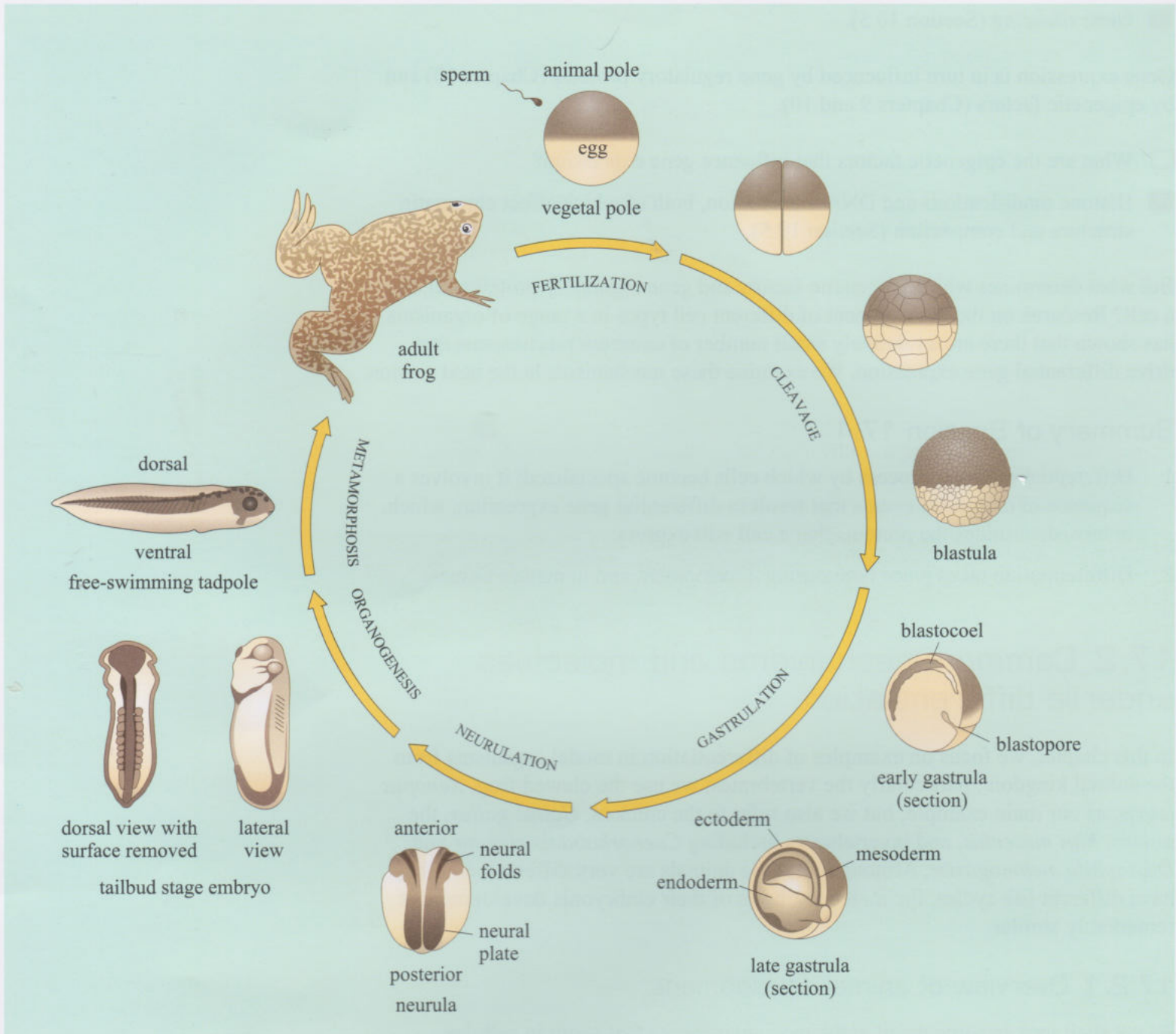


Figure 17.2 The main stages in the development of *Xenopus laevis*. The unfertilized egg is not uniform; the animal and vegetal poles differ in content and appearance. After fertilization, cell division during the cleavage stage results in the formation of a fluid-filled blastula. Gastrulation follows, during which inward movement of cells results in the formation of the three germ layers (endoderm, mesoderm and ectoderm) and the specification of the anterior–posterior (head–tail) axis (not shown) and the neural plate. During neurulation, the neural tube forms from the neural plate and body segmentation begins. Organogenesis (formation of the body organs) is followed by metamorphosis (the transformation of the tadpole into a frog). Note that the representations of the different embryonic and developmental stages are not drawn to scale.

The egg and fertilization

Factors that drive cellular differentiation are in place in the egg even before fertilization occurs, and after fertilization important developmental events take place before the first division. The entry of sperm triggers *activation* of the egg. In all animals, this activation involves a rapid increase in the concentration of calcium ions, which propagates as a *calcium wave* (Section 13.3.3) across the egg and is essential for subsequent changes within the egg that are needed for development to proceed (Figure 17.3). We cannot detail all these changes here, and once again, they vary from species to species, but they include metabolic activation, DNA synthesis and sometimes increases in protein synthesis. The importance of the increase in calcium ion concentration after fertilization has been demonstrated in experiments in which calcium levels are raised artificially in unfertilized sea-urchin oocytes. All the events of fertilization are triggered in these oocytes, but the process almost always ceases before the first cell division, because the artificially activated cell lacks the sperm pronucleus (the name given to the sperm nucleus when it has entered the egg) and associated centriole, which is needed for mitosis (Chapter 8, Section 8.2.3).

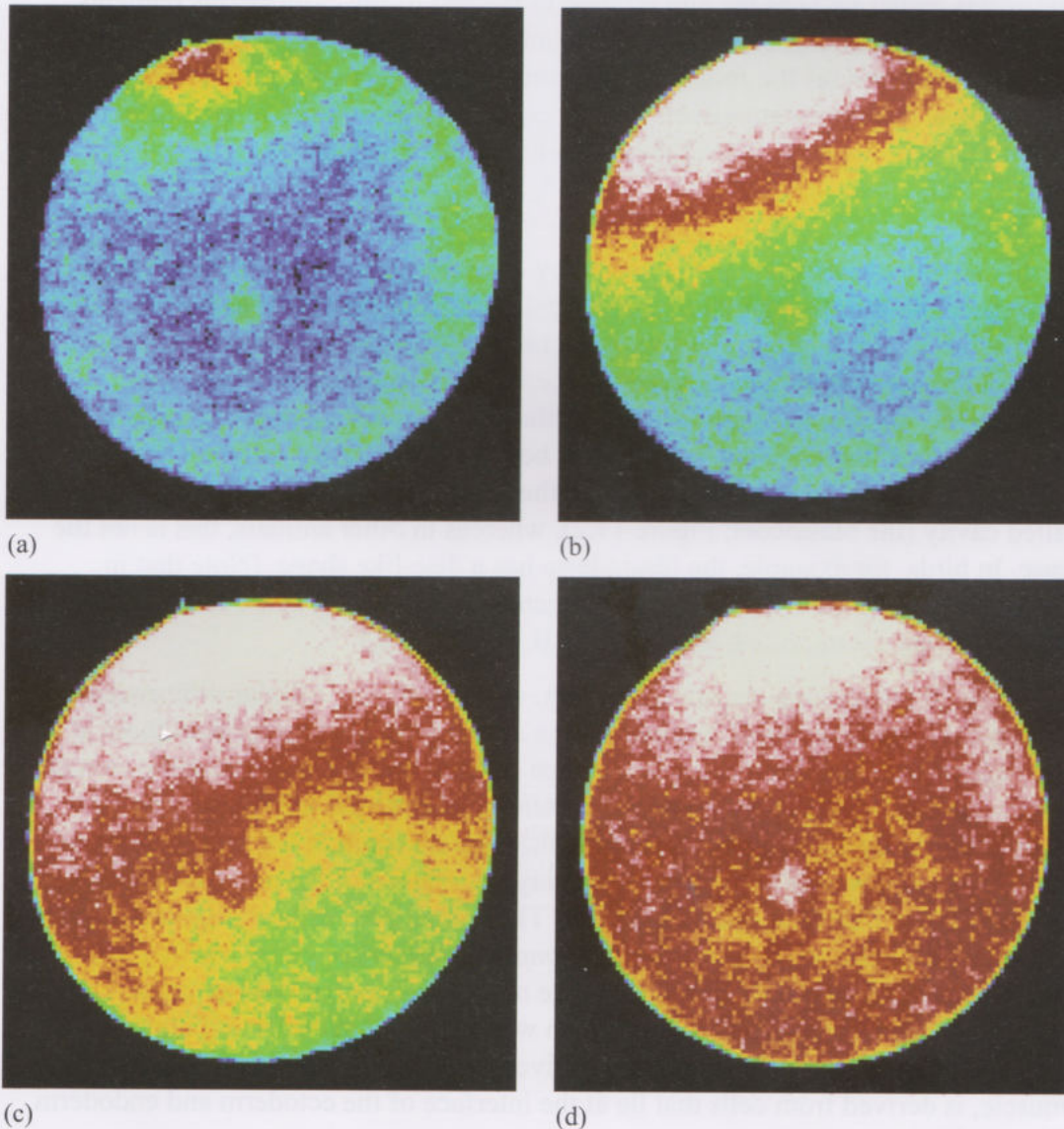


Figure 17.3

Confocal light micrograph showing different stages of a calcium wave in a fertilized sea-urchin oocyte. (a) An initial local increase in the calcium concentration occurs at the site of sperm entry (top left). (b) and (c) The calcium increase is subsequently propagated to the rest of the oocyte in the form of a calcium wave. (d) The fertilized oocyte shows a generalized increase in calcium concentration across the entire cytosol. Increasing calcium levels are represented using the following colour scale (in ascending order): blue < green < yellow < red < white.

- Calcium chelators are molecules that bind and sequester calcium ions, and hence prevent their action. What would be the effect of injection of a calcium chelator into a fertilized egg?
- The processes of metabolic activation and DNA synthesis would be inhibited, thereby preventing the normal development of the embryo.

The egg contains a mixture of proteins and also of mRNA, which are derived from the mother. The mRNA present in the unfertilized egg is therefore known as **maternal mRNA**. In some animals, such as amphibians, these proteins and mRNAs are not uniformly distributed within the egg, so after fertilization and the first division of the zygote, the two daughter cells contain different combinations of proteins and mRNAs.

The dorsal side of an animal is its back, whereas the ventral side is the animal's belly. The dorso-ventral axis is therefore the line that runs between the back surface and the undersurface of the animal.

The anterior part of an animal is its head whereas the posterior is its tail. The anterior-posterior axis is therefore the line that runs from the head to the tail of the animal and is generally perpendicular to the dorso-ventral axis.

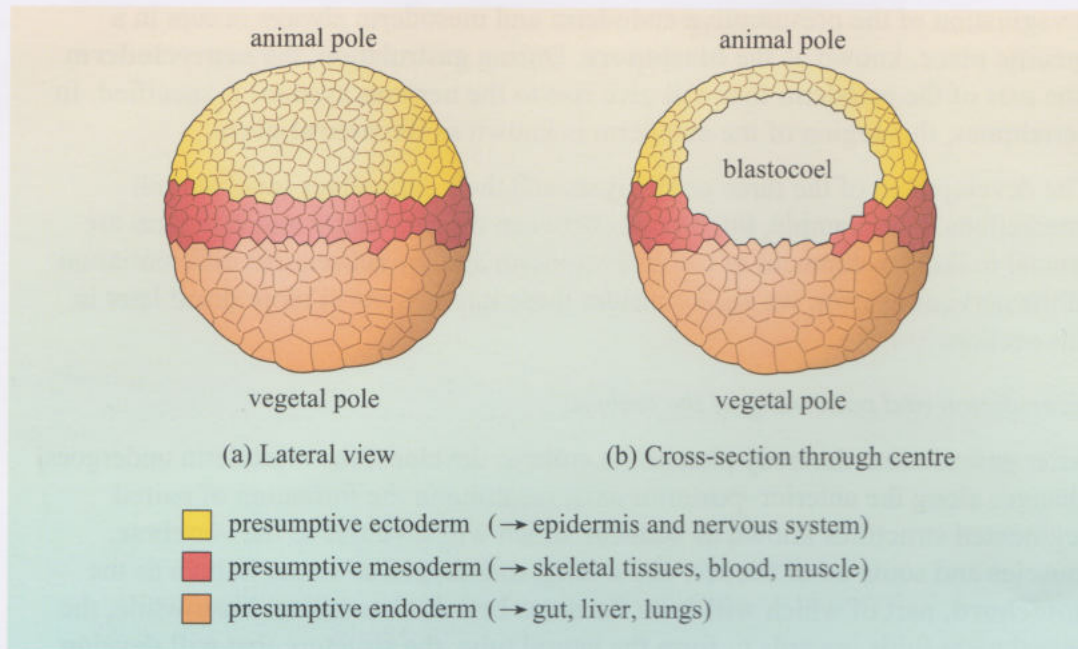
Note that these axes are *specified* in early embryos, before polarity is established.

Unfertilized frog eggs have a pigmented animal pole and paler vegetal pole, which contains mostly yolk proteins. The yolk proteins are dense, so the animal pole, which contains little yolk, lies uppermost (Figure 17.2). Fertilization occurs at the animal pole, and as well as resulting in fusion of the sperm and egg pronuclei (involving events that we do not describe here), causes a rotational movement of the cortex (outer part) of the egg relative to the rest of the cytoplasmic contents, an event known as cortical rotation. This movement plays a role in differentiation, because it determines the future dorso-ventral axis of the embryo. The site of sperm entry has important effects on subsequent differentiation in some other species as well as frogs. We return to this topic later (Section 17.2.3).

Cleavage

Fertilization and activation are followed by **cleavage** (Figure 17.2), the division of the zygote to produce a group of smaller cells known collectively as the **blastula** (or blastocyst in mammals, blastoderm in birds). Cleavage divisions are so called because they occur without cell growth (i.e. there is no increase in size, so successively smaller cells are formed). Although the resulting group of cells is often spherical, there are many variations between blastulas of different species. In amphibians and certain other animals, the cells of the blastula surround a fluid-filled cavity (the blastocoel; Figure 17.2), whereas in other animals, this is not the case. In birds, for example, the blastoderm has a disc-like shape. (Note that in *Drosophila*, only the nuclei divide during cleavage, resulting in the formation of a syncytial blastoderm; S204, Book 3, Vol. II, Chapter 10.)

The cells of the blastula are not equivalent, as you will see; cellular differentiation begins at cleavage. In frogs, the cells at the animal pole are smaller than those at the vegetal pole and, because of the uneven distribution of the cytoplasmic contents of the egg, they also contain different proteins and maternal mRNA species. The early differences that arise in the cells of the blastula result in the specification of cells that will give rise to the three **germ layers**, regions of the embryo that will develop into different tissues in the adult. The animal pole cells will go on to form the **ectoderm** (and so are known as *presumptive* ectodermal cells) which gives rise to the epidermis (skin) and cells of the nervous system, while the vegetal pole cells will form the **endoderm**, which will form the gut, liver and lungs. The third germ layer, the **mesoderm**, which gives rise to skeletal tissues, blood and muscle, is derived from cells that lie at the interface of the ectoderm and endoderm (Figure 17.4).

**Figure 17.4**

Specification map of cells in the frog blastula. Cells from the animal pole will give rise to ectoderm; cells in the vegetal pole will give rise to endoderm. Cells in the equatorial region will give rise to the mesoderm. (a) Lateral surface view. (b) Cross-section view through the centre of the blastula, showing the blastocoel, a fluid filled cavity that lies under the animal pole.

Gastrulation

The germ layers are formed during the next stage, which is known as **gastrulation** (from the Greek *gaster*, meaning 'belly'), shown in Figure 17.2. During gastrulation, the presumptive endoderm and mesoderm move to the inside of the embryo, and the ectoderm spreads around the outside (Figure 17.5). Gastrulation involves dramatic cell movements, and results in the formation of inner, middle and outer tissue layers, and the specification of the anterior–posterior (head–tail) axis. (One way to picture gastrulation of the frog embryo is to imagine a balloon that is not quite fully inflated, so that is possible to push one side of the balloon in until it meets the other side – a simple invagination is formed.)

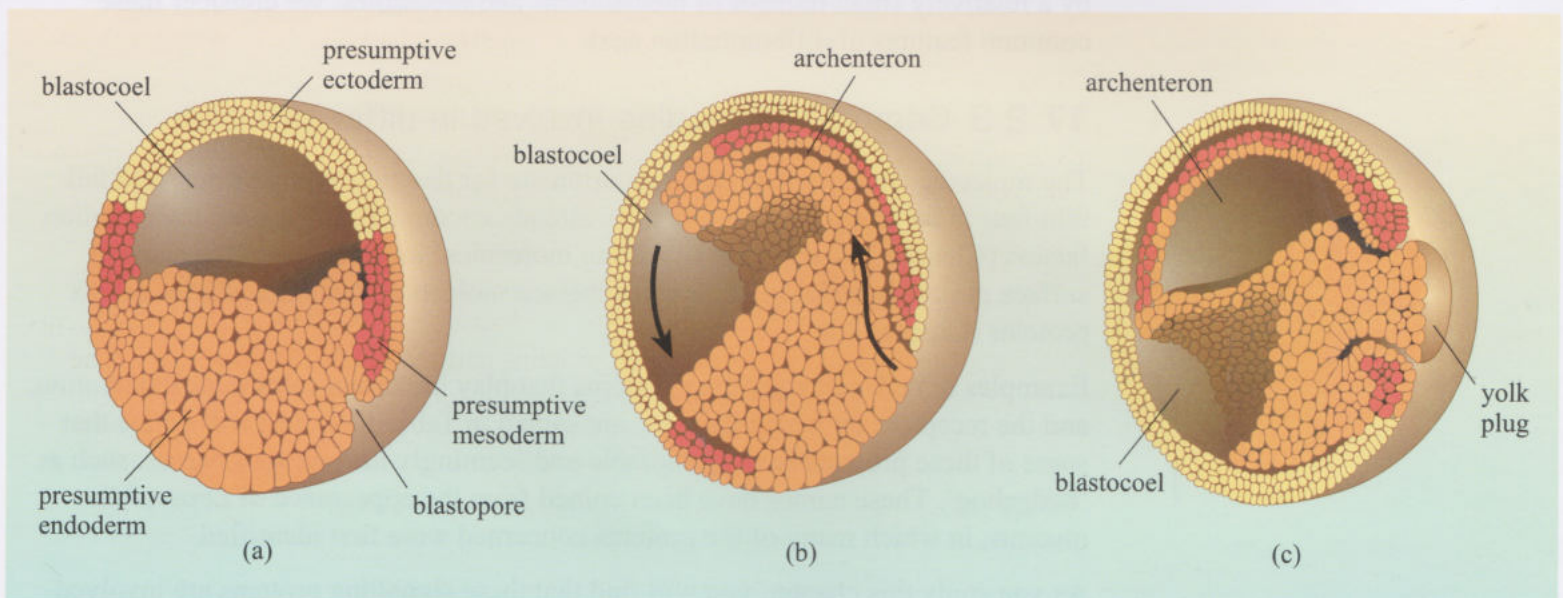


Figure 17.5 Gastrulation in *Xenopus* – cross-section views of gastrulas at different stages are shown. (a) Invagination of presumptive endoderm and mesoderm begins at a region known as the blastopore. (b) Presumptive endoderm and mesoderm stream inwards (black arrows), so that the ectoderm comes to lie at the outside of the gastrula. A new cavity, the archenteron, which goes on to develop into the gut, is formed. (c) The anterior–posterior (A–P) axis is specified and the blastocoel becomes smaller.

Invagination of the presumptive endoderm and mesoderm always occurs in a specific place, known as the **blastopore**. During gastrulation, the **neurectoderm** (the part of the ectoderm that will give rise to the nervous system) is specified. In vertebrates, this region of the ectoderm is known as the **neural plate**.

The development of the three germ layers and their derivatives involves cell interactions. For example, interactions between the mesoderm and ectoderm are crucial to the development of the neurectoderm and the subsequent differentiation of the nervous system; we shall consider these interactions in more detail later in this section.

Neurulation and patterning of the embryo

After gastrulation, the body plan of the embryo develops; the mesoderm undergoes changes along the anterior–posterior axis, resulting in the formation of paired segmented structures known as **somites** which will give rise to the vertebrae, muscles and some other tissues, and a long, rod-shaped structure known as the **notochord**, part of which will form the areas between vertebrae. Meanwhile, the neural plate folds inwards to form the neural tube, the structure that will develop into the brain and spinal cord of the adult animal. This process is known as **neurulation** (Figure 17.6).

Organogenesis and metamorphosis

The next stage in development of most animals is **organogenesis**, i.e. the formation of the different organs, such as the limbs, heart, gut and kidney. In amphibians and some other animals, such as insects, a larval form develops, and metamorphosis (from the Greek, meaning ‘transformation’) into an adult often involves both formation of new organs, and loss of others, such as the tadpole tail, the cells of which die by apoptosis (Chapter 14, Figure 14.1).

The wide range of cellular changes, rearrangements and movements that take place during the development of different organs in different animals are brought about by a relatively small number of mechanisms and molecules. We consider these common features of differentiation next.

17.2.3 Common molecules involved in differentiation

The molecules that regulate differentiation are for the most part proteins, and fall into four main groups, which you have already encountered. They are transcription factors (Chapter 10), cell–cell signalling molecules (including secreted and cell surface molecules; Chapter 13), cell adhesion molecules and extracellular matrix proteins (Chapter 16).

Examples of cell–cell signalling proteins that play important roles in differentiation, and the receptors on which they act, are shown in Table 17.1. You will notice that some of these proteins have memorable and seemingly incongruous names, such as ‘hedgehog’. These names have been coined from the appearance of *Drosophila* mutants, in which many of the proteins concerned were first identified.

As you study this chapter, you will find that these signalling proteins are involved repeatedly during different stages of development, and at different places in the developing embryo.

Patterning is a general term used to describe the formation of an ordered pattern of cellular organization and activity.

The nervous system is subdivided into the central nervous system, which consists of the brain and spinal cord, and the peripheral nervous system, which consists of small groups of neurons known as ganglia located at various places in the body.

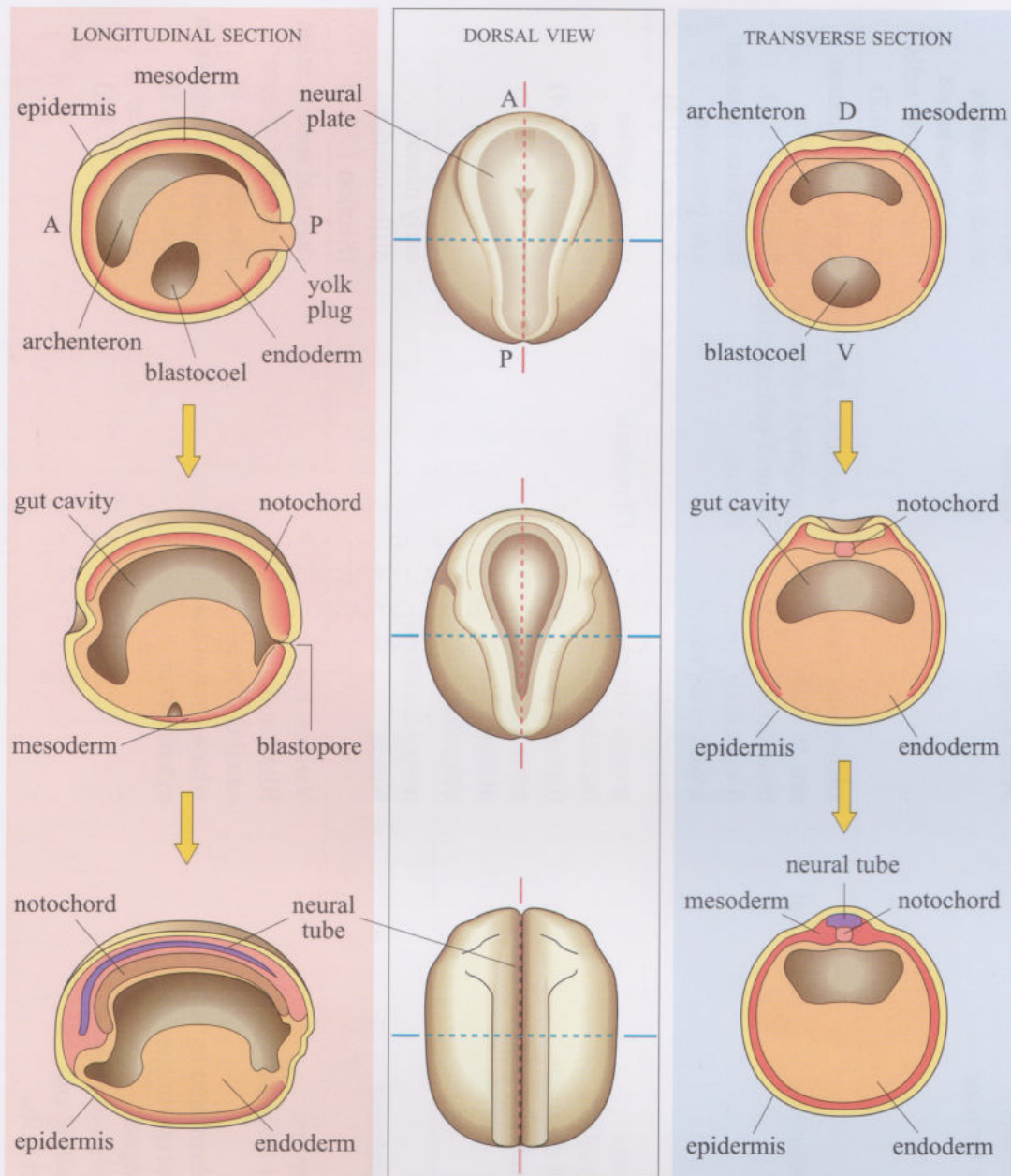


Figure 17.6 Neurulation of the amphibian embryo. Three views at different stages of neurulation are shown. Left column: a cross-sectional view along the length of the middle of the embryo (longitudinal section along the plane determined by the anterior–posterior axis). A = anterior pole; P = posterior pole. Middle column: a surface view of the embryo, from the dorsal side, showing the folding of the neural plate, which begins at the anterior region. Right column: a cross-sectional view across the middle of the embryo (transverse section along the plane determined by the dorso-ventral axis). D = dorsal pole; V = ventral pole. The planes of the sections are shown on the surface view.

Before we turn to look at some of the mechanisms that are also repeatedly used in cellular differentiation, we review how the processes and molecules responsible for differentiation and development are studied. Some of the main techniques that are used are described in Box 17.1.

Table 17.1 Examples of some common cell-cell signalling proteins in differentiation.

(Note that you do not need to learn all the terms in this table.)

Cell-cell signalling molecules		Receptors	Endogenous inhibitors	Downstream mediators	Transcription factors directly activated	Role(s) in cellular differentiation
Family	Examples					
TGF β superfamily (secreted polypeptides)	bone morphogenetic proteins (BMPs), Vegenin (Vg), Activin, Nodal	TGF receptors, Type I and II (receptor serine–threonine kinases)	Noggin, Follistatin	Smads 1, 2, 3 and 5 (named from Sma in <i>C. elegans</i> and Mad in <i>Drosophila</i>)	Smads 1, 2, 3 or 5 complexed with Smad 4	mesoderm induction and patterning (Section 17.2.4); motor neuron specification (Section 17.3.4)
FGF family (secreted polypeptides)	20+ members (e.g. FGF-2)	FGF receptors (receptor tyrosine kinases)		MAP kinases	Various	involved in many processes, e.g. maintenance of neural stem cells (Section 17.4.2)
Wnt family (secreted cysteine-rich glycoproteins)	15 members in vertebrates (named from <i>Drosophila</i> wingless, <i>Wg</i> , and its vertebrate homologue, <i>integrated</i>)	Frizzled (7TM receptors)	Frzb, Dickkopf	Dishevelled (which acts to inhibit breakdown of free β -catenin by the action of a protein complex)	β -catenin, LEF/TCF (lymphoid enhancer factor/T cell specific factor)	early specification in <i>C. elegans</i> (Section 17.2.3); mesoderm induction and patterning (Section 17.2.4)
hedgehog family (secreted polypeptides)	vertebrate homologues of <i>Drosophila</i> hedgehog: sonic (Shh), desert (Dhh) and Indian hedgehog (Ihh)	patched and smoothened (12- and 7TM receptors respectively)		protein complex includes Ci (cubitus interruptus) protein; in absence of signal, Ci is ubiquitinated	Ci protein	motor neuron specification (Section 17.3.4)
Delta (transmembrane protein)		Notch (transmembrane protein)		protease cleaves Notch		early neural differentiation (Section 17.3.2)
ephrins (cell surface, transmembrane, or soluble extracellular proteins)	ephrin-A class (GPI-linked); ephrin-B class (transmembrane domain)	Eph receptors (receptor tyrosine kinases) <i>Note</i> : ephrin binding to Eph receptors can initiate signal transduction in both the signalling and the target cells.		Various, e.g. Rho-family small GTPases which control the cytoskeleton		regional specification in nervous system (Section 17.3.3); neural crest migration (Section 17.3.5); axon guidance (Section 17.3.6)

Box 17.1 How is cell differentiation studied?

Cell differentiation is studied by a variety of methods, including analysis of gene expression, cellular ablation, transplantation, genetic analysis, cell tracing and cell culture techniques. Here we outline these methods, illustrating their use with examples, some of which will be described in more detail later in this chapter.

Analysis of gene expression

Differential gene expression underlies the differences between cell types, and the process of differentiation involves sequential changes in gene expression. The techniques for analysis of gene expression that you have already encountered, such as *in situ* hybridization and microarray analysis (Chapter 5, Box 5.1), are therefore widely used to determine which genes are expressed by developing cells and tissues.

- ☐ What important advantage does *in situ* hybridization offer over other methods of analysis of gene expression?
- ☒ It allows analysis of gene expression at the *single cell* level.

Cell ablation

The selected destruction or removal (ablation) of specific cells has been widely used in the study of early development and also in the later differentiation of invertebrate cells during development, especially in *C. elegans* and *Drosophila melanogaster*. An example of this technique is the ablation, by laser microsurgery, of specific cells in the early *C. elegans* embryo (see Section 17.2.3).

Cell tracing

One of the ways of studying the differentiation of a cell is to mark or label it in some way, so that its fate can be followed during later stages of development. Substances that are fairly stable and can be readily detected, such as rhodamine- or fluorescein-conjugated dextran, which are too large to cross cell membranes, are widely used. Such tracers are microinjected into cells. In early embryos, for example, labelling of individual blastomeres (cells of the blastula) with such tracers allows their progeny to be identified later in development. Results from many such experiments allow construction of a **fate map**, which shows the normal fate of cells in particular regions of an early embryo (you will see an example of a fate map in Figure 17.12, Section 17.2.4).

Cell labelling and tracing is also used in the study of the fate of migratory cells, such as the neural crest cells (Chapter 16, Figure 16.1) that give rise to several different types of cell in the fully developed animal, including neurons of the peripheral nervous system. We consider this topic in more detail in Section 17.3.4.

Transplantation

The transplantation of groups of cells from one region of a developing embryo to another has been widely employed in the study of differentiation. The method is used to find out if cells in particular regions of early embryos are already committed to a specific fate, or if they can adopt the fate of the cells in the region to which they are transplanted. The transplanted cells are usually labelled in some way – for example, with tracers as described above – to distinguish them from the host cells. An alternative method is to perform *xenografts* (grafts between different species) between two closely related species that have cells with properties that allow them to be distinguished. Examples are chicks and quails; quail nuclei can be distinguished from those of chicks because of the distinctive arrangement of their heterochromatin.

Genetic analysis

Genetic analysis of mutant animals that exhibit developmental abnormalities has provided a wealth of invaluable information about how differentiation is regulated. As in the case of cell death, the organisms most studied are *C. elegans* and *D. melanogaster*. You will encounter many examples of the use of this powerful approach for the study of differentiation later in the chapter.

Cell culture

Cell culture is a widely used technique (which you have come across already in *Experimental investigations 2* and *3* to investigate protein compartmentalization and signal transduction pathways, respectively). Cells from animals at all ages can be grown *in vitro*, when provided with a suitable nutrient-containing culture medium, an appropriately adhesive surface (substratum) on which to attach, and incubated at physiological temperatures. Early embryos and also explants (small pieces) of differentiated tissues can be maintained *in vitro*, until they become too large for gases and nutrients to diffuse to the centre of the tissue. Often experiments are performed on ‘dissociated’ cell cultures, i.e. cultured cells that have been separated from their neighbours.

An example of the use of cell culture is when cells of an early embryo, such as cells from different regions of the blastula, are separated and grown in a so-called basal medium (one that has only nutrients and no instructive factors). The fate of the cells grown in these minimal conditions can be analysed. This information is then used to create a **specification map** (Figure 17.4), which differs from a fate map in that it provides information about the potential of the cells in different parts of the embryo at the stage at which they are cultured.

One of the main applications of cell culture is in the analysis of the effects of exogenous factors on the properties of the cultured cells. For example, factors that are believed to be involved in normal growth and/or differentiation can be added to the culture medium at a range of concentrations, and their effects on cellular parameters such as cell proliferation, differentiation and cell death can be measured. The downstream effects on the molecular properties of the cultured cells, such as gene expression, can also be analysed.

17.2.3 Common mechanisms in differentiation

Asymmetric division

When a cell divides, its components are normally evenly distributed between the two daughter cells, which are therefore nearly identical. However, an unequal distribution of proteins or mRNAs between the two daughters can differentially influence the fate of the two cells. This type of division is known as **asymmetric division**, and it plays an important role in the differentiation of cells. Note that asymmetric division can result in the production of cells that are either unequal or equal in size.

- ☐ What structure brings about cytokinesis in animal cells?
- ☒ The contractile ring, which is composed of actin and myosin filaments, along with other proteins (Chapter 8, Section 8.2.6).
- ☐ What determines the position of the contractile ring?
- ☒ The arrangement of the astral and spindle microtubules.

Asymmetric division is involved in the development of all animals, but perhaps the two best-studied examples are the generation of neural precursors from neural stem cells in the developing nervous system and the early development of *C. elegans*. In Chapter 1, you saw that the lineage of every cell in the adult *C. elegans* is known. Here we briefly revisit this lineage, shown in more detail for the early stages of development in Figure 17.7.

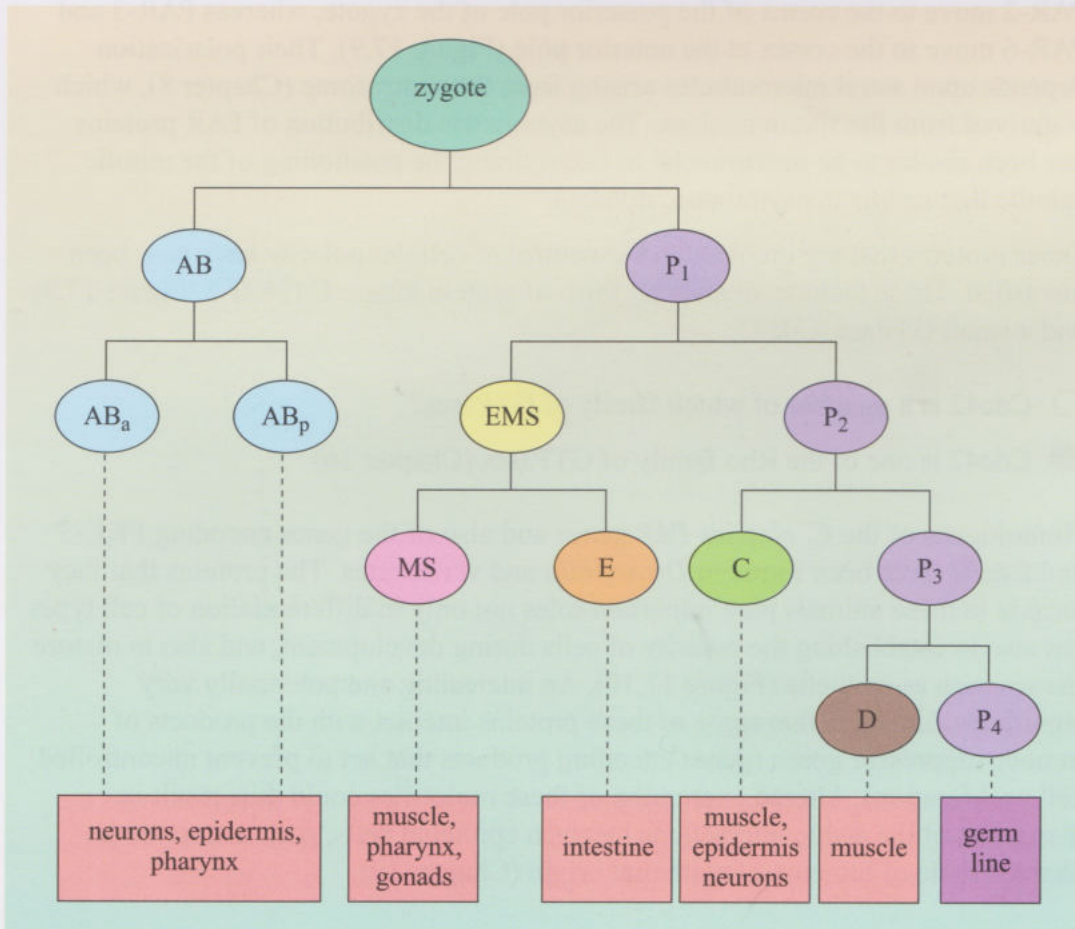


Figure 17.7

The early lineage of *C. elegans*, showing the formation of five founder cells (AB, MS, E, C and D) that give rise to the differentiated cells shown, and a stem cell lineage (originating from the posterior cell, P₁) that gives rise to the germ cell line.

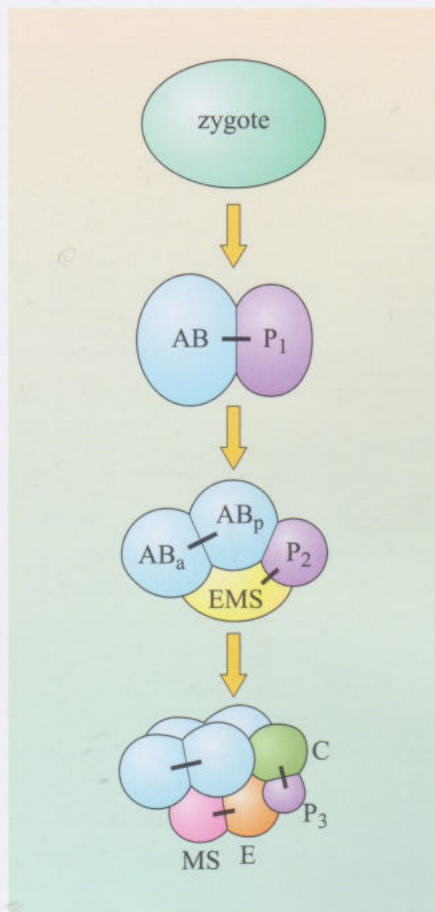


Figure 17.8

The first three cleavage divisions of *C. elegans*, showing asymmetric and symmetric divisions. Cells formed by asymmetric division are illustrated in different colours; those arising by symmetric division are shown in the same colour. Sister cells are indicated by connecting bars.

PKC-3 is atypical because its activity is *not* regulated by calcium ions or DAG (Section 13.3.2).

During early cleavage of *C. elegans*, asymmetric division plays a key role in the formation of so-called founder cells, whose progeny give rise to differentiated cells of the adult, and a stem cell lineage that will form the germ line.

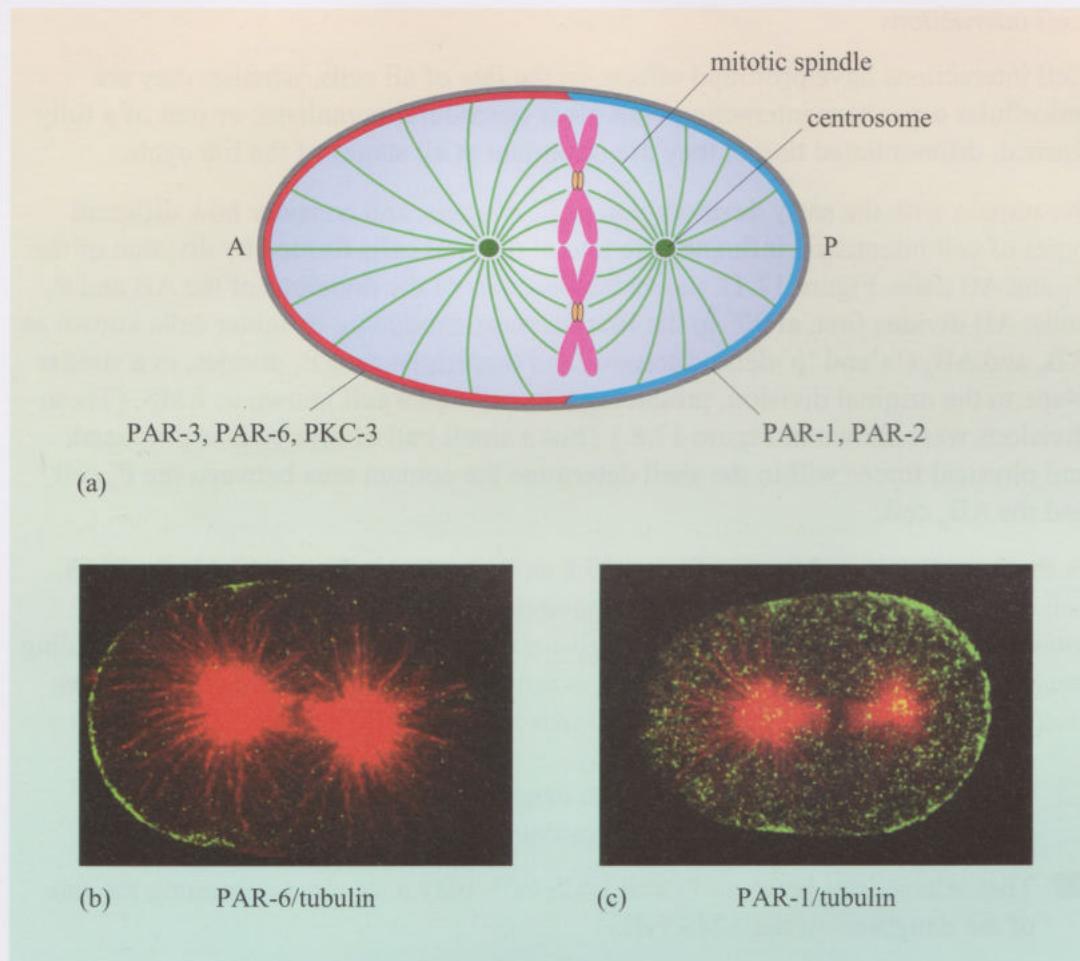
The asymmetric divisions that occur in early cleavage of *C. elegans* are essential to subsequent development; when this predictable and ordered pattern of division is disrupted, normal development is also affected. What regulates these asymmetric divisions? The nucleus of the unfertilized egg is non-centrally located, but otherwise the egg appears to be symmetrical. Yet the very first division of the zygote is unequal, and results in the production of one large and one smaller cell, as shown in Figure 17.8. It turns out that the position at which this first division occurs is determined by the site of entry of the sperm, and determines the polarity of the embryo. The point of sperm entry will become the posterior of the developing embryo, and division occurs nearest that pole, so that the posterior (P_1) cell is smaller than the anterior (AB) cell (Figure 17.8). The daughter cells that arise from this first division are not only different in size, but also differ in their components.

How asymmetric cell divisions occur is not yet fully understood, but some of the molecules involved and their modes of action have been identified. The first step is the entry of the sperm, which triggers cytoplasmic rearrangements of a group of maternally derived proteins, encoded by the maternal *PAR* (from *partitioning defective*) genes. These genes were identified by analysis of mutant worms in which early cell divisions were abnormal. There are at least six *PAR* genes, and the proteins they encode are diverse. However, all *PAR* proteins are localized to the cortex of the cell, and are involved in regulation of the distribution of other cytoplasmic components within cells during early development, by mechanisms that involve the cytoskeleton. After fertilization, but before the first division, *PAR*-1 and *PAR*-2 move to the cortex of the posterior pole of the zygote, whereas *PAR*-3 and *PAR*-6 move to the cortex at the anterior pole (Figure 17.9). Their polarization depends upon astral microtubules arising from the centrosome (Chapter 8), which is derived from the sperm nucleus. The asymmetric distribution of *PAR* proteins has been shown to be instrumental in determining the positioning of the mitotic spindle that results in asymmetric division.

Other proteins that are involved in the control of cellular polarity have now been identified. These include an atypical form of protein kinase C (PKC-3, Figure 17.9) and a small GTPase, Cdc42.

- ☐ Cdc42 is a member of which family of GTPases?
- ☒ Cdc42 is one of the Rho family of GTPases (Chapter 16).

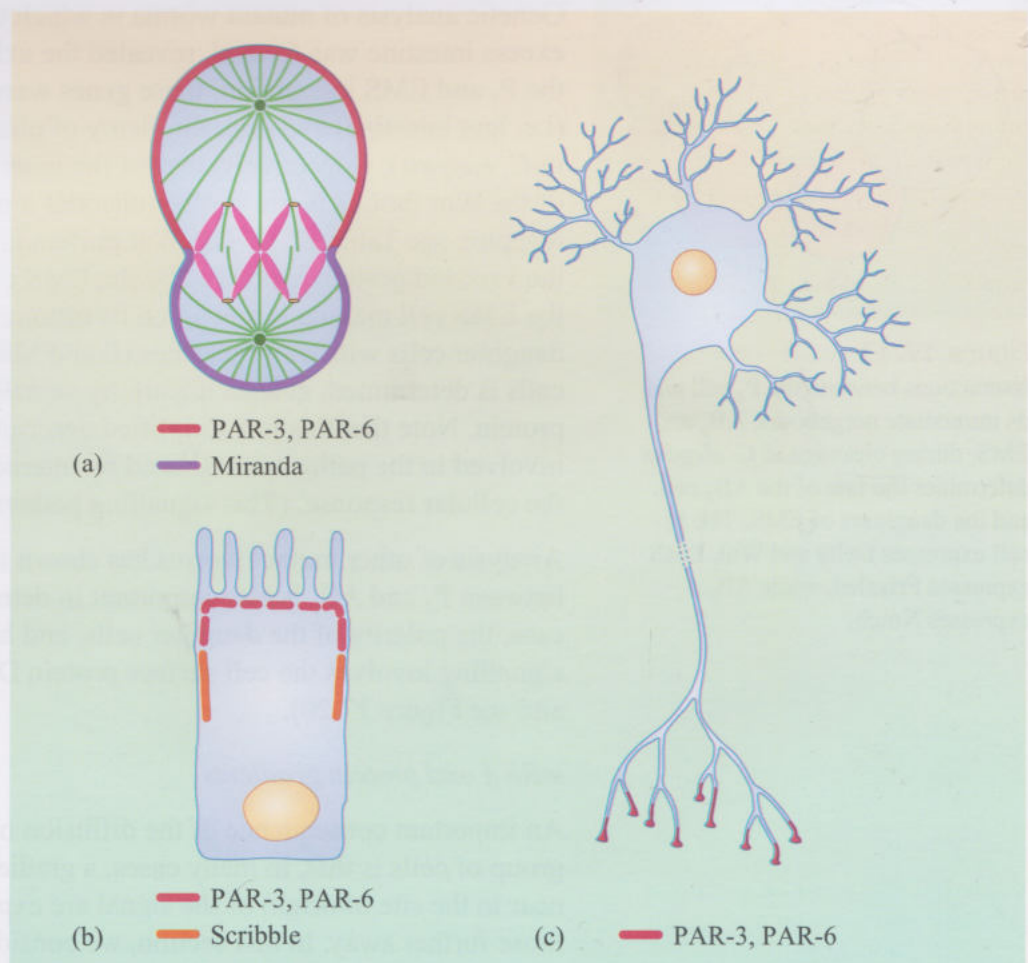
Homologues of the *C. elegans* *PAR* genes and also of the genes encoding PKC-3 and Cdc42 have been found in *Drosophila* and vertebrates. The proteins that they encode in these animals play important roles not only in differentiation of cell types but also in establishing the polarity of cells during development, and also in mature tissues such as epithelia (Figure 17.10). An interesting and potentially very significant finding is that some of these proteins interact with the products of tumour suppressor genes (genes encoding products that act to prevent uncontrolled cell proliferation). Altered expression of these molecules could thus result in disruption of the stable interactions between epithelial cells; such disruption is characteristic of tumours of epithelial origin (Chapter 19).

**Figure 17.9**

Localization of PAR proteins and PKC-3 in the *C. elegans* zygote. (a) Cell polarity in the *C. elegans* zygote. The cortex is divided into distinct anterior (A) and posterior (P) domains. PAR-3, PAR-6 and PKC-3 (red shading) are localized in the anterior cortex, whereas PAR-1 and PAR-2 (blue shading) are restricted to the posterior cortex. The mitotic spindle is positioned closer to the posterior pole, which results in the generation of a larger anterior and a smaller posterior cell after cytokinesis. Astral microtubules emanate from the centrosomes and reach the cortex. (b) Double-label immunofluorescence image of a wild-type *C. elegans* zygote stained with anti-PAR-6 (green) and anti-tubulin (red) antibodies. (c) Double-label immunofluorescence image of a wild-type *C. elegans* zygote stained with anti-PAR-1 (green) and anti-tubulin (red) antibodies. (Source: Wodarz, 2002) (The role of tubulin, which forms microtubules in mitosis, was described in Section 8.2.3.)

Figure 17.10

PAR proteins play a role in the polarization of different cell types. The localization of PAR proteins is polarized and influences the distribution of other proteins within these cells. (a) *Drosophila* neural precursors, known as neuroblasts, undergo asymmetric division to give rise to another neuroblast (larger cell) and a ganglion mother cell (smaller cell) that will differentiate into two neurons. The protein Miranda, which plays a role in the uneven distribution of proteins that influence the fate of the cells, is localized to the opposite pole to PAR-3 and PAR-6. (b) Epithelial cells have distinct apical and basolateral regions (Book 1, Figure 6.35). This polarity is established by a number of proteins, including a PDZ domain protein known as Scribble, which is involved in the formation of adherens junctions. The localization of the PAR proteins to the apical region results in a more lateral localization of Scribble, and thus plays a role in determining the positioning of adherens junctions and the polarity of the cell. (c) In a developing neuron, expression of PAR-3 and PAR-6 is localized to the tips of growing axons, where they may play a role in the reorganization of the cytoskeleton.



Cell interactions

Cell interactions have profound effects on the fate of all cells, whether they are unicellular organisms interacting with other unicellular organisms, or part of a fully formed, differentiated tissue; they are important at all stages of the life cycle.

The EMS cell is the progenitor cell of the E and MS blastomeres. It was given this abbreviated name because it gives rise to the endoderm, mesoderm and stomodeum (part of the embryonic gut).

We remain with the early development of *C. elegans*, and consider how different types of cell interaction influence the fate of the first cells formed by division of the P_1 and AB cells. Figure 17.11 shows the products of the divisions of the AB and P_1 cells; AB divides first, at 90° to the first division, producing daughter cells known as AB_a and AB_p ('a' and 'p' denote anterior and posterior); then P_1 divides, in a similar plane to the original division, producing a P_2 cell and a cell known as EMS. (These divisions were shown in Figure 17.8.) Thus a small ball of four cells is produced, and physical forces within the shell determine the contact area between the P_2 cell and the AB_p cell.

In the next division, shown in Figure 17.8 and repeated in Figure 17.11, the EMS cell gives rise to the E cell (which will give rise to the endodermal cells of the intestine) and the MS cell (which will give rise to mesodermal derivatives, including muscle) (see Figure 17.7). If the P_2 cell is removed by microsurgery (cell ablation; Box 17.1), the EMS cell divides to give two MS cells.

- ☐ What does this change of fate of the daughters of the EMS cell upon P_2 removal show about the mechanisms determining their differentiation?
- ☒ That interactions between P_2 and EMS cells play a role in determining the fate of the daughters of the EMS cell.

Genetic analysis of mutant worms in which either no intestinal cells developed, or excess intestine was formed, revealed the existence of genes that were involved in the P_2 and EMS interaction; these genes were called *mom*, for more mesoderm (i.e. less intestine) and *pop*, for plenty of pharynx (the pharynx is part of the foregut in *C. elegans*), respectively. One of the *mom* genes was found to encode a protein of the Wnt family, while another encodes a protein related to Frizzled (a Wnt receptor, see Table 17.1). The Wnt protein is expressed by the P_2 cell, and acts on the Frizzled protein expressed by the EMS cell (Figure 17.11). This signal polarizes the EMS cell making it reposition its mitotic spindle thereby generating two daughter cells with different fates (E and MS). Thus the fate of the EMS daughter cells is determined, at least in part, by signalling mediated by Wnt, a secreted protein. Note that this is a simplified description; several other molecules are also involved in the pathways activated by interaction between Wnt and Frizzled and in the cellular response. (This signalling pathway is summarized later in Figure 17.15.)

Analysis of other mutant worms has shown that contact-mediated signalling between P_2 and AB_p is also important in determining the fate of AB_p ; in this case, the polarity of the daughter cells, and hence of the embryo, is affected. This signalling involves the cell surface protein Delta acting on Notch (Table 17.1, and see Figure 17.20).

mRNA and protein gradients

An important consequence of the diffusion of a soluble protein from a cell or small group of cells is that, in many cases, a gradient of the signal is set up, so that cells near to the site of origin of the signal are exposed to higher levels of the signal than those further away. In this section, we consider the role of protein gradients and also mRNA gradients in differentiation.

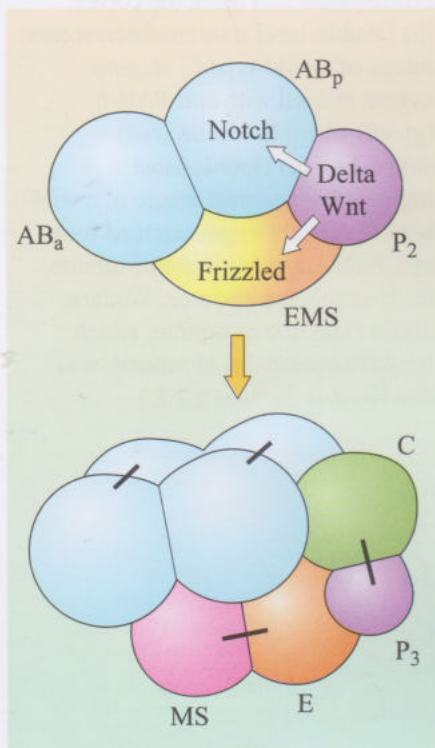


Figure 17.11

Interactions between the P_2 cell and its immediate neighbours, AB_p and EMS, during cleavage in *C. elegans* determines the fate of the AB_p cell and the daughters of EMS. The P_2 cell expresses Delta and Wnt. EMS expresses Frizzled, while AB_p expresses Notch.

In some animals, the distribution of mRNA and protein within the unfertilized egg is uneven, or there may be a gradient of maternal mRNA within the egg. In addition to the *Xenopus* oocyte, another example that you have already encountered is the *Drosophila* egg, in which *bicoid* mRNA is localized at the anterior pole (Figure 10.42c). When fertilization occurs, this mRNA is translated, and a gradient of bicoid protein is set up. As you have seen, proteins can have different effects at differing concentrations, particularly if they are regulatory proteins. Bicoid is a transcription factor, and influences transcription of the genes of the zygote (zygotic genes). So, the gradient of bicoid creates a different pattern of gene expression in different parts of the *Drosophila* embryo. Such maternal genes, which exert their effects in the zygote and early embryo, are known as **maternal effect genes**, and they play an important role in the early stages of differentiation (Section 17.4.2).

Maternal effect genes, such as *bicoid*, and protein gradients were introduced in S204, Book 3 *The Core of Life*, Vol. II, Section 10.2.1 and mentioned again in S377, Book 2, Chapter 10.

This simple mechanism, the creation of a protein or mRNA gradient which leads to exposure to different levels of regulatory molecules, is used widely and repeatedly during different stages of development and in different places in the embryo. Gradients of secreted proteins may be created by diffusion; thus different cells are exposed to differing levels of a signalling molecule. Such gradients are important both in the early stages of development, and also later, when they play an important role in **morphogenesis** (the change in form that occurs during development), and so are known as **morphogen gradients**.

- ☐ Can you think of a problem with the mechanism of simple diffusion as a means of creating a stable gradient of a signalling molecule?
- ☒ Over time, all cells will be exposed to the same amount of the signalling molecule, unless there is some mechanism to stabilize the gradient.
- ☐ Suggest mechanisms that would aid in the creation of a gradient of a signalling molecule.
- ☒ (1) The signalling molecule could break down rapidly, or be degraded by the cells that bind it/take it up. (2) There may be an opposing gradient of a molecule that neutralizes, or has the opposite effect to, the signalling molecule. (3) The molecule may move selectively, rather than by simple diffusion. For example it may be bound to the extracellular matrix, or it may be transported from one cell to another.

An example of a gradient not created by diffusion is the transport gradient established by transcytosis of the Wnt family member Wg, which is important during the development of *Drosophila* (Section 12.2.5). You will meet examples of other mechanisms that aid in the creation of signalling gradients later in this chapter.

Combinatorial control

What should have become clear during this section is that as development and differentiation proceed, cells are exposed to a range of signals, at different concentrations and at appropriate times. The response of a cell at any time depends upon the nature, levels and combination of the different molecules it is exposed to.

- ☐ What type of proteins influence how a cell responds to an extracellular signalling molecule?
- ☒ Cell surface receptors and components of the downstream intracellular signalling cascades (Chapter 13).

Simultaneous or sequential activation of different signalling pathways will result in the activation or inhibition of different transcription factors, and hence the transcription of different genes. Since a cell is exposed to a number of signalling molecules at any time, its response will be the result of the combined action of these signalling pathways and hence the combined action of the transcription factors that they activate, a phenomenon known as **combinatorial control**.

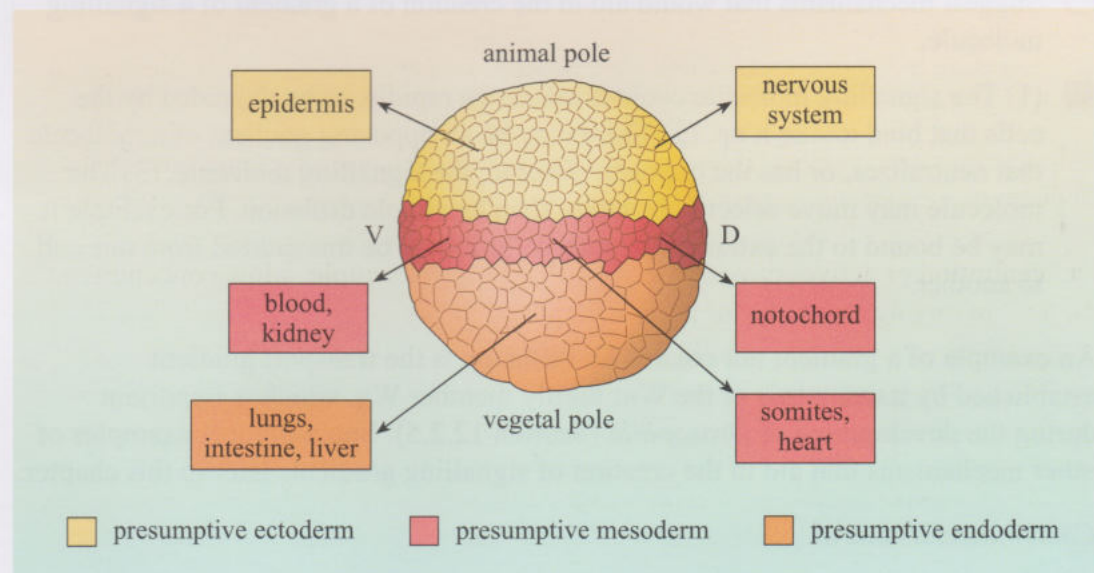
17.2.4 Mesoderm induction involves gradients and combinatorial control

A good example of the common mechanisms and molecules in the early stages of differentiation is in induction of the mesoderm in *Xenopus* embryos. **Induction** is a change in the properties of a group of cells that occurs as a result of signals from a nearby group of cells. You have seen that the mesoderm is the germ layer that will form from the cells that lie at the interface between the animal and vegetal poles of the blastula (Figure 17.4). Experiments in which animal and vegetal pole cells were grown together in culture (co-culture) has shown that a mesodermal fate is induced in cells of the animal cap (part of the animal pole) by a factor produced in the vegetal pole cells. Evidence from researchers in several laboratories showed that a member of the TGF β (transforming growth factor β) family of secreted proteins was likely to be this factor.

This description, however, presents a simplified picture, as the cells at different positions along the dorso-ventral axis of the presumptive mesoderm give rise to different types of differentiated cells, as shown in Figure 17.12. Moreover, in co-culture experiments, different regions of the presumptive endoderm were found to induce different cell types from animal cap cells (Figure 17.13).

Figure 17.12

Fate map of the *Xenopus* blastula (lateral view). Cells in different regions of the presumptive germ layers will give rise to different specialized cell types. The presumptive ectoderm gives rise to epidermal tissues and the nervous system. The presumptive mesoderm gives rise to somites and notochord – which are transient embryonic structures that will form muscle and skeletal tissues – and also to blood and kidney. Cells of the presumptive endoderm gives rise to the lungs, liver and intestine.



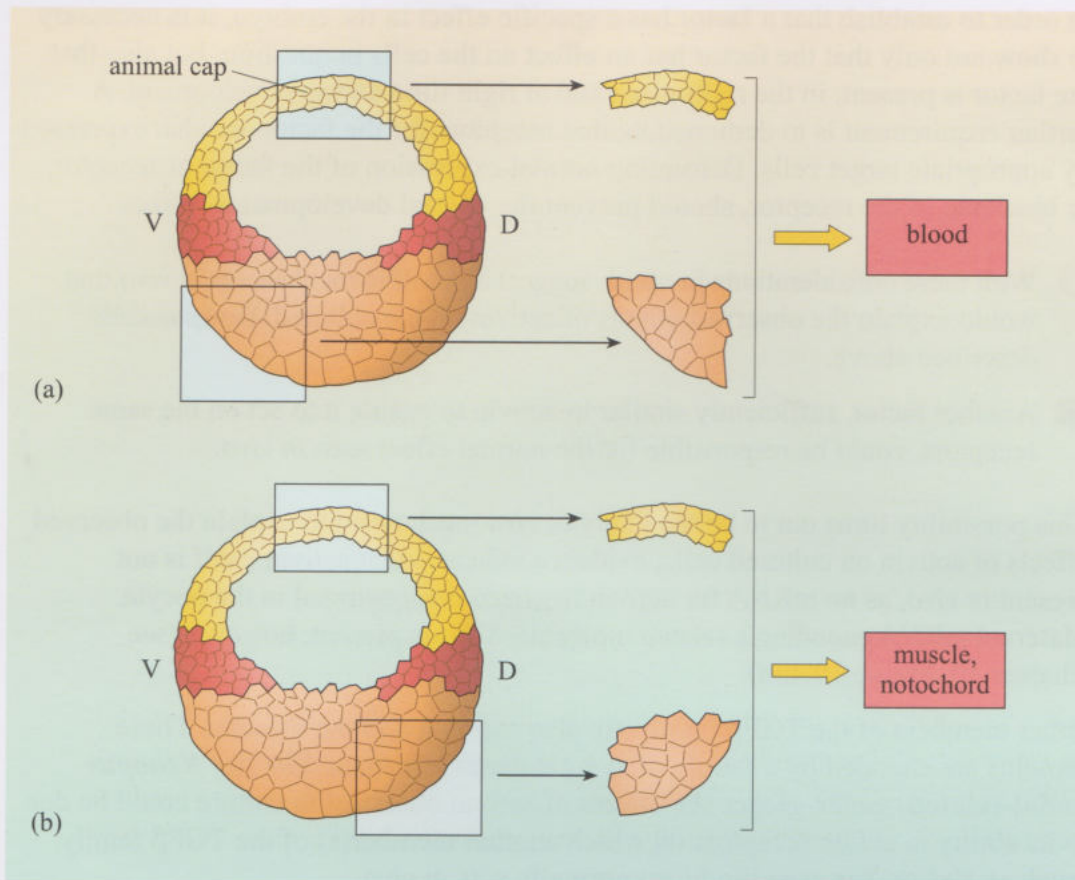


Figure 17.13

Different regions of the presumptive amphibian endoderm induce differentiation of different mesodermal cell types in co-culture with animal cap cells. Cross-section view of blastulas (from *Xenopus*) highlighting the areas taken for co-culture experiments. (a) Co-culture of animal cap plus presumptive ventral endoderm stimulates differentiation into blood. (b) Co-culture of animal cap with presumptive dorsal endoderm promotes differentiation into muscle and notochord.

- ☐ Suggest two possible explanations for the induction of different cell types in the animal cap by different regions of the endoderm in culture.
- ☒ Different inducing signals could be produced by the dorsal and ventral endoderm, or there could be a gradient of a signalling protein along the dorso-ventral axis of the endoderm. A third possibility is that both these mechanisms are involved in patterning the mesoderm.

One hypothesis for the molecular mechanism that results in the dorso-ventral patterning of the presumptive mesoderm is that a gradient of a signalling protein is produced by the vegetal pole cells. This is illustrated by a TGF β family member in Figure 17.14.

For several years, the TGF β family member **activin** was a strong contender as the inducer of mesoderm. Experiments in the 1990s showed that activin has differential effects on dispersed *Xenopus* animal cap cells grown in culture, depending upon the concentration of activin provided to the cells. So, for example, a low concentration of activin (0.1 ng ml^{-1}) caused the cells to differentiate into blood-like cells and mesenchymal (loose connective tissue-like) cells, whereas a substantially higher concentration (10 ng ml^{-1}) promoted differentiation into notochord cells. Cells that were not exposed to activin differentiated into epidermis-like cells.

- ☐ Do the experiments on activin described above prove that this growth factor is responsible for determining the differentiation of mesodermal cells *in vivo*?
- ☒ No, they only show what effect activin is capable of having on these cells *in vitro*.

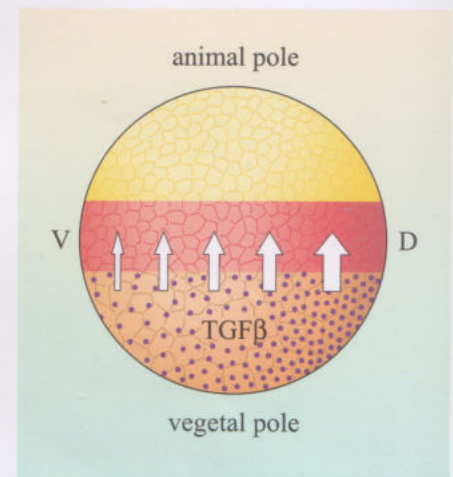


Figure 17.14

Simple model for the dorso-ventral patterning of the presumptive mesoderm of the frog blastula. A gradient of signalling molecules belonging to the TGF β family (depicted as dots) is produced by the cells of the endoderm along the dorso-ventral axis. TGF β diffuses to the presumptive mesoderm, so cells in the dorsal region of the presumptive mesoderm are exposed to higher concentrations of this signal than cells in the ventral region.

In order to establish that a factor has a specific effect in the embryo, it is necessary to show not only that the factor has an effect on the cells in question, but also that the factor is present, in the right place and at right time during development. A further requirement is to demonstrate that receptors for the factor are also expressed by appropriate target cells. Disrupting normal expression of the factor or receptor, or blockade of the receptor, should prevent the normal developmental effect.

- With these considerations in mind, suggest a possible mechanism *in vivo* that would explain the observed effects of activin on the cultured *Xenopus* cells, described above.
- Another factor, sufficiently similar to activin to enable it to act on the same receptors, could be responsible for the normal effect seen *in vivo*.

This possibility turns out to be the likely *in vivo* mechanism to explain the observed effects of activin on cultured cells; evidence indicates that activin itself is not present *in vivo*, as no mRNA for activin has been demonstrated in the oocyte. Maternal mRNA encoding a related molecule, *Vg1*, is present, however (see Chapter 10, Figure 10.42b).

Other members of the TGF β family are also expressed in the blastula. These proteins are encoded by a family of zygotic genes known as *Xnr* (for *Xenopus nodal-related*) genes. Hence the effects of activin observed in culture could be due to its ability to act on receptors on which another member(s) of the TGF β family, (such as *Vg1* or *Xnr* gene products) normally acts *in vivo*.

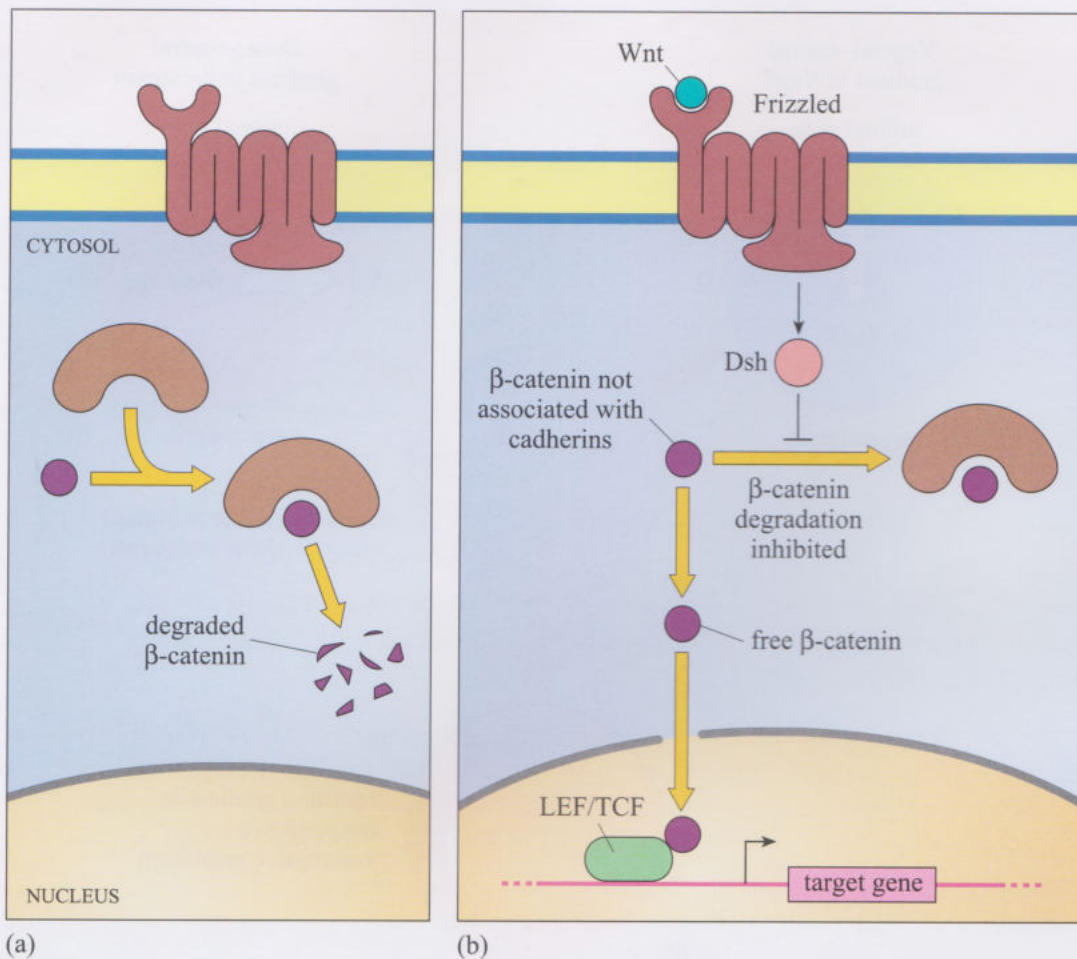
Expression of the *Xnr* genes is induced by the maternally provided transcription factor, **VegT**, which is expressed in cells of the vegetal pole. So, how is a gradient of Xnr-related proteins along the dorso-ventral axis set up? It is the result of the combined action of VegT and another transcription factor, **β -catenin**, which also promotes Xnr expression, as described below.

β -Catenin is a multifunctional protein; it not only acts as an adaptor linking cadherins to actin in cell–cell junctions (Chapter 16), but is also a final downstream component of the Wnt–Frizzled signalling pathway, shown in Figure 17.15. Proteins that activate this pathway were identified independently in mice and *Drosophila* (Table 17.1). Proteins of the Wnt family act on 7TM receptors (Chapter 13) of the Frizzled family. The most common mechanism of action of the Frizzled receptors differs from that of most other such receptors, as it does not involve G proteins, but instead involves a cytosolic messenger protein called **Dishevelled (Dsh)**.

When the Wnt–Frizzled pathway is inactive, any β -catenin that is not associated with cadherins (see Chapter 16) is broken down (Figure 17.15a). This breakdown is the result of the action of a complex of proteins that we do not detail here, but includes one known as APC (adenomatous polyposis coli, a tumour suppressor protein; mutations in the *APC* gene occur in approximately 80% of human colon cancers – Section 19.9). When Wnt proteins bind to Frizzled receptors, Dsh is activated, and inhibits the breakdown of β -catenin by the complex. The spared β -catenin is therefore able to translocate to the nucleus, where it complexes with, and acts as a co-activator with the transcription factor **LEF/TCF** (lymphoid enhancer factor/T cell specific factor), shown in Figure 17.15b and listed in Table 17.1. Although the details of this pathway are still being elucidated, it is of interest to note that the mutations resulting in inactivation of the *APC* gene that occur in human colon cancer lead to an increased transcriptional activity by β -catenin–LEF/TCF.

The APC protein should not to be confused with the anaphase promoting complex (APC), Chapter 8.

LEF/TCF should not to be confused with ternary complex factor (TCF), Section 13.3.7.

**Figure 17.15**

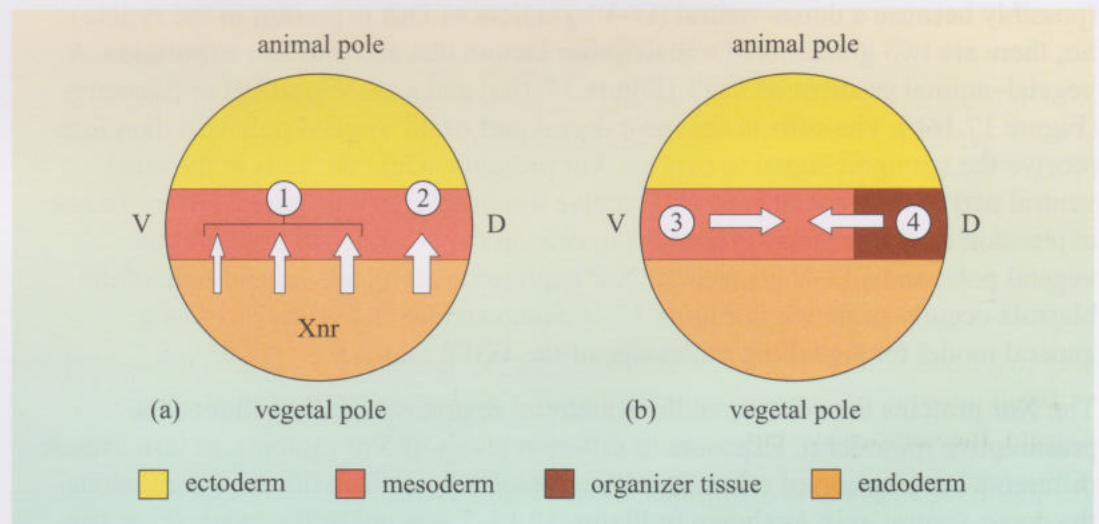
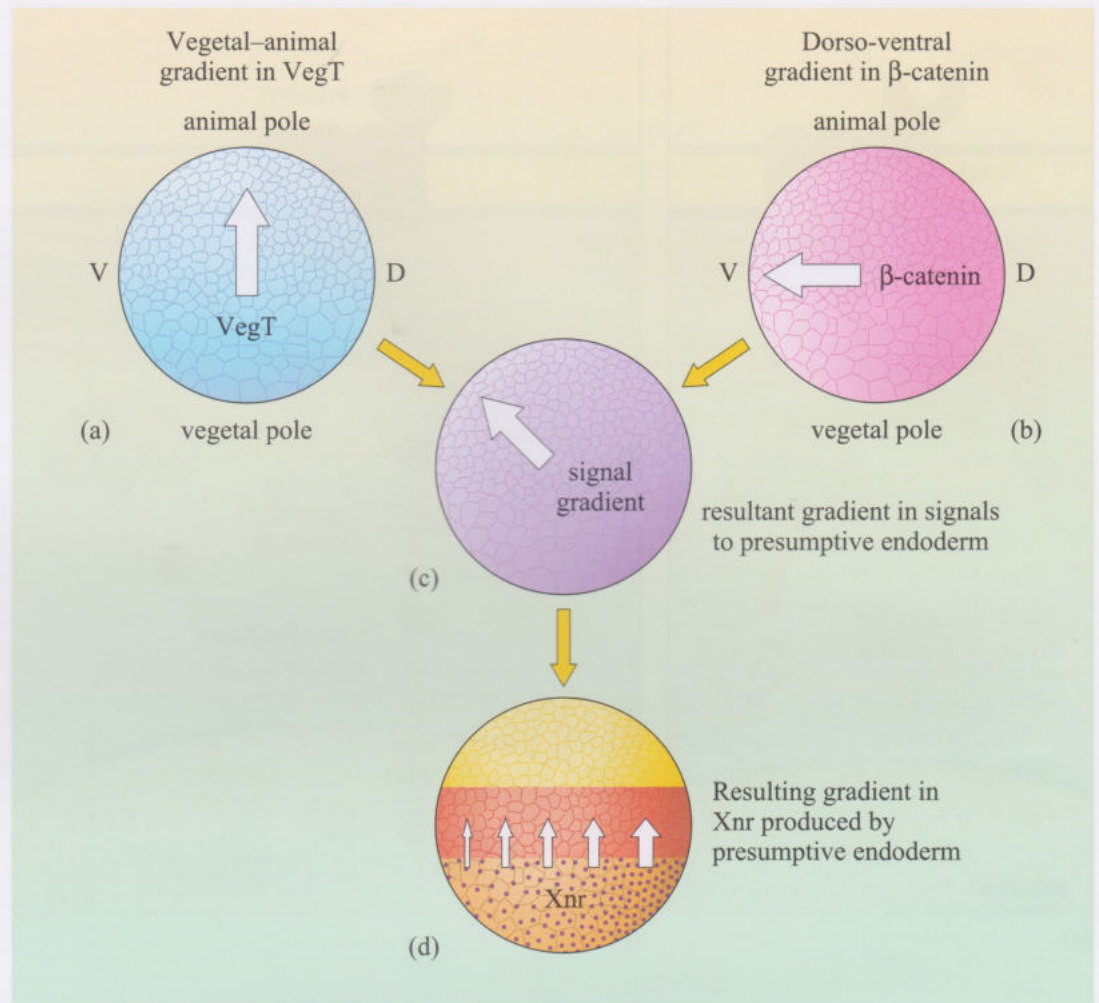
The Wnt–Frizzled signalling pathway. In (a) The Wnt–Frizzled pathway is not activated and β -catenin is degraded by a complex of proteins which includes APC (light brown). In (b) binding of Wnt to Frizzled activates Dishevelled (Dsh) which inhibits β -catenin degradation. Free β -catenin translocates to the nucleus and, together with LEF/TCF, initiates transcription of target genes. This is a simplified diagram, and does not include all components of the pathway.

β -Catenin is expressed throughout the zygote, but is only stabilized in dorsal regions (possibly because a dorso-ventral (D–V) gradient of Dsh is present in the zygote). So, there are two gradients of transcription factors that activate Xnr expression. A vegetal–animal gradient of VegT (Figure 17.16a) and a D–V gradient of β -catenin (Figure 17.16b). The cells in the most dorsal part of the vegetal pole will therefore receive the strongest signal to express Xnr proteins, while the cells in the most ventral part of the vegetal pole will receive a weaker signal (Figure 17.16c). Hence expression of Xnr proteins is greatest in cells in the most dorsal region of the vegetal pole, and a D–V gradient of Xnr expression along the vegetal pole of the blastula occurs, as shown in Figure 17.16d (shown also in Figure 17.14 as a general model for signalling molecules of the TGF β family).

The Xnr proteins then diffuse to the equatorial region, where they induce the presumptive mesoderm. Exposure to different levels of Xnr proteins, in turn causes differential expression of other signalling proteins by cells in the mesoderm along the dorso-ventral axis, as shown in Figure 17.17. Exposure to lower levels of Xnr proteins in the ventral mesoderm stimulates production of proteins of the BMP (bone morphogenetic protein) and Wnt families (see Table 17.1). Exposure of cells in the most dorsal part of the mesoderm to high levels of Xnr proteins stimulates expression of a different group of secreted signalling proteins by these cells. These are inhibitory proteins and they play an important role in later development, as will be described in Section 17.3.1.

Figure 17.16

The transcription factors VegT and β -catenin act together to produce a gradient in Xnr expression, which in turn produces a dorso-ventral gradient of Xnr proteins along the presumptive *Xenopus* mesoderm. (a) VegT mRNA is localized in cells of the vegetal pole and so a vegetal–animal gradient of VegT protein is set up. (b) β -Catenin expression is stabilized in the dorsal region, creating a D–V gradient of transcriptional activity. (c) shows the resultant gradient of combined signals and (d) shows that VegT and β -catenin together promote expression of Xnr genes, which are hence expressed in a gradient along the D–V axis, and most highly at the most dorsal region of the presumptive endoderm.

**Figure 17.17** A model for signals that induce and pattern the *Xenopus* mesoderm.

(a) Induction of the mesoderm. A gradient in the expression of Xnr proteins is set up along the dorso–ventral axis. The highest level of protein is produced in the dorsal part of the vegetal pole (2); lower levels are produced more ventrally (1). (b) Patterning of the mesoderm. Low and intermediate levels of Xnr proteins stimulate expression of BMPs and Wnt (not shown) in the cells of the ventral mesoderm (3). The high level of Xnr proteins in the dorsal vegetal pole induces production of a fourth signal (4), a group of signalling proteins produced by the cells of the most dorsal part of the mesoderm, called the organizer (see Section 17.3.1).

Recall that the end point of many signalling pathways is the activation (or inactivation) of transcription factors. The combination, levels and time of activation of transcription factors within a cell will determine the nature of the genes that it expresses. For example, the transcriptional activity of VegT and β -catenin together in the cells of the most dorsal part of the mesoderm leads to the expression of other transcription factors, which in turn stimulate transcription of other regulatory genes, initiating a cascade of transcription factors, or of genes that encode proteins essential to later development.

Summary of Section 17.2

- 1 Animal development involves a series of overlapping processes. Cleavage divisions, which form the blastula, are followed by gastrulation, during which the three germ layers (ectoderm, mesoderm and endoderm) are formed and specification of the neurectoderm and the anterior–posterior axis take place. The neural tube forms during neurulation, and body segmentation begins. Finally organogenesis and in some species, metamorphosis, occur.
- 2 Differentiation involves molecules that are evolutionarily conserved between animal species, and act in different places and at different times during animal development. These molecules include transcription factors, and a number of different secreted and cell surface signalling proteins, their receptors and downstream intercellular signalling molecules. Differential exposure to signalling molecules results in differential activation of transcription factors, leading to differential gene expression.
- 3 Differentiation is studied by cell ablation, cell tracing, transplantation, genetic analysis and cell culture techniques.
- 4 Similar mechanisms are involved in differentiation of different cells and tissues, of all animal species studied. These mechanisms include asymmetric division, cell interactions, protein and mRNA gradients and combinatorial control.
- 5 Induction and patterning of the mesoderm, like that of other embryonic tissues, involves protein and mRNA gradients and combinatorial control.

17.3 Differentiation of the nervous system during development

Developmental biology is a vast subject. Clearly, we cannot attempt to describe the differentiation of all types of cells here; rather we have chosen to focus on a single system, the nervous system.

Of all the organ systems of animals, the nervous system provides the greatest challenge to developmental cell biologists. Estimates suggest that the mammalian nervous system consists of 10^{14} neurons and also different types of ‘supporting’ cells known as glial cells. The neurons are of many different types, form complex circuits, and receive specialized inputs (via synapses, of which there are approximately 10^{23} in the mammalian brain) from many other neurons. Other animals, however, have smaller and simpler systems; *C. elegans*, for example, has 302 neurons. As in the case of cell death (Chapter 14), the analysis of the molecules and mechanisms involved in the development of these systems has provided fundamental information that has helped our understanding of differentiation in more complex nervous systems.

In this description of nervous system development, we have selected examples, focusing on neuronal differentiation in vertebrates. To begin, we outline the main stages involved. Note that the development of the nervous system also includes processes that we do not describe here, such as cell death (Chapter 14) and neurochemical differentiation; the latter determines which of many possible neurotransmitter molecules a neuron will produce and use to signal to its target cells. Differentiation of glial cells is also a crucial aspect of nervous system development that we do not describe.

- ☐ After formation of the three main germ layers, what is the first stage in the segregation of cells that are destined to become neurons?
- ☒ Specification of the neurectoderm (Section 17.2.2).

The first step in the differentiation of cells of the nervous system is the specification of part of the ectoderm into neurectoderm, which forms a sheet of cells with epithelial properties (the neuroepithelium) and is known as the neural plate (Figure 17.6). This process depends upon interactions between cells of the future neurectoderm and other cells, and is called **neural induction**; it will be discussed in Section 17.3.1.

Although the neurectodermal cells are specified, they are undifferentiated; their differentiation into neurons is dependent upon transcription of regulatory genes, which is, in turn, dependent upon asymmetric divisions and combinatorial control exerted by diffusible and contact-mediated signals. We describe the signals that ‘make a neuron’ in Section 17.3.2.

Once the neurectoderm has been specified, morphogenesis of the nervous system begins.

- ☐ In vertebrates, what is the first step in the morphogenesis of the nervous system?
- ☒ Neurulation, which results in the formation of the neural tube, which will give rise to the brain and spinal cord (Section 17.2.2).

After the neural tube closes, patterning of the nervous system to form the brain and spinal cord begins. The fate of the cells in these different regions is dependent upon positional information; Hox genes play an important role in this process, as described in Section 17.3.3.

There are many different types of neuron, which differ not only in their neurochemical properties, but also in their shapes and projections. We consider some aspects of how different types of neuron are specified in Section 17.3.4.

The cells of the neural tube retain their epithelial properties until they begin to differentiate, apart from a particular group of cells known as **neural crest cells**. These cells separate from the neuroepithelium and migrate from the dorsal edge of the neural tube to distant parts of the developing embryo, where they give rise to neurons and glial cells of the peripheral nervous system, as well as some other cell types. This process is described in Section 17.3.5.

One of the defining features of neurons is their complex morphology, which enables them to make multiple connections with cells that may be – in large mammals, for example – several metres from their cell body. Developing neurons

extend long processes known as axons (see Figure 17.10c), which form precise connections with their target cells; often this entails complex trajectories. We consider the process of **axon guidance** in Section 17.3.6.

17.3.1 Neural induction

Neural induction has been a topic that has exercised developmental biologists for many years, and it continues to be a controversial subject. You have seen that a specific part of the presumptive ectoderm is destined to develop into neurectoderm, and the remainder will differentiate into epidermal tissues (Figure 17.12). What are the signals that induce a neural fate in this particular group of cells? Classical early experiments by the embryologists Spemann and Mangold (1924) using amphibians showed that a particular part of the blastula was needed for nervous system formation. This region was the dorsal lip of the blastopore, which is the site where, during gastrulation, invagination begins, as you saw in Figure 17.5a. Recall also that the most dorsal part of the mesoderm is the site of production of the fourth signal that patterns the mesoderm (Section 17.2.4, Figure 17.17).

Spemann and Mangold grafted segments of early gastrulas between two newt species that differed in pigmentation, so the host and donor cells could be readily distinguished. Grafts of the dorsal lip of the blastopore, to the opposite side of an equivalent early gastrula resulted in the formation of an additional body axis in the developing host embryo, with a second neural tube. Spemann and Mangold called this dorsal lip region the **organizer**, and their discovery began the search for the substance(s), produced by the organizer, that were believed to induce the neurectoderm.

Later experiments, however, provided some unexpected evidence about the process of neural induction. Instead of secreting proteins that stimulated the first stages of a program of neural differentiation, the organizer was found to secrete proteins that inhibited differentiation of the ectodermal cells into epidermal cells. The first such protein to be identified was named **Noggin**, and it was found to be expressed during gastrulation; first by the cells of the dorsal lip of the blastopore, then by those of the notochord. Noggin acts by binding to and thereby inactivating BMP proteins (Table 17.1).

Soon after the identification of Noggin, other related molecules were identified that also promoted formation of neurectoderm, and inhibited BMPs (these proteins included Follistatin and Chordin). Thus a model for neural induction in amphibians was that signals from the organizer induce neurectoderm by repressing the action of members of the BMP family, as shown schematically in Figure 17.18.

You saw in Section 17.2.4 that another secreted protein, a Wnt family member, is produced in the non-dorsal mesoderm (Figure 17.17). This protein inhibits the formation of the neurectoderm on the ventral ectoderm. The organizer also secretes proteins that inhibit Wnt signalling; one of these proteins is Frzb (pronounced 'Frisbee'). So, the organizer tissue produces proteins that inhibit the actions of both BMPs and Wnt, and it is thought that this inhibitory action suppresses differentiation into epidermal cells, thereby promoting neural induction and the formation of neurectoderm.

The term *process* is often used to describe the parts of a cell that project from the cell body.

Hans Spemann, from Freiburg, won the Nobel Prize for Physiology or Medicine in 1935 for his discovery of the organizer effect. Note that Spemann was an established embryologist who had performed much pioneering work leading to the discovery. Mangold, as the junior scientist, was only involved in part of the work, and was directed by Spemann, so was not awarded a share of the Prize.

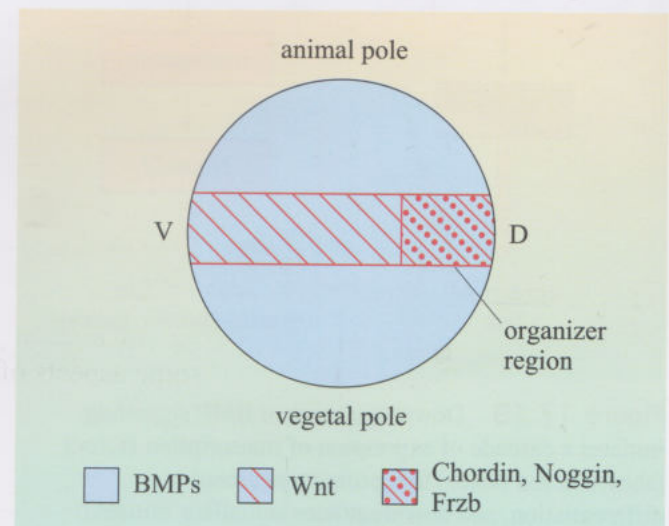


Figure 17.18 Model for induction of the amphibian neurectoderm by inhibitors of BMPs and Wnt produced by the organizer region. BMPs are expressed throughout the embryo; Wnt is expressed along the D–V axis (with high levels on the ventral side). The organizer tissue produces proteins such as Chordin, Noggin and Frzb that inhibit the action of BMPs and Wnt.

The cellular arrangement of the gastrula of other vertebrates, such as birds and mammals, differs somewhat from that of amphibians, but these other vertebrates also have regions that appear to act in a similar way to the amphibian organizer. Evidence from birds and mammals suggests that, in these species at least, the first events in neural induction may take place before gastrulation, and that the animal cap cells may receive an early signal that makes them competent to become neural.

- ☐ Suggest what might make cells competent to respond to a signalling protein.
- ☒ Expression of appropriate receptors and downstream intracellular signalling proteins, which are necessary for a cell to be able to respond to a particular extracellular (secreted or contact-mediated) signalling protein. The expression of these proteins would, in turn, depend upon the expression of the appropriate combination of transcription factors.

Although much remains to be learnt about neural induction, downregulation of BMP signalling seems to be a key event common to all vertebrates; the effect of this downregulation is outlined in the next section.

17.3.2 Contact-mediated signals upregulate expression of neuron-specific transcription factors

The reduction in BMP expression in the presumptive neurectodermal cells is pivotal to subsequent neural differentiation. BMP has two important effects; it stimulates expression of the transcription factor GATA-1, which promotes epidermal fate, and

it inhibits transcription of genes that regulate neuronal fate. So the downregulation of BMP in neurectodermal cells relieves the inhibition of neurogenic gene expression, and initiates a cascade of expression of further transcription factors, some of which are shown in Figure 17.19.

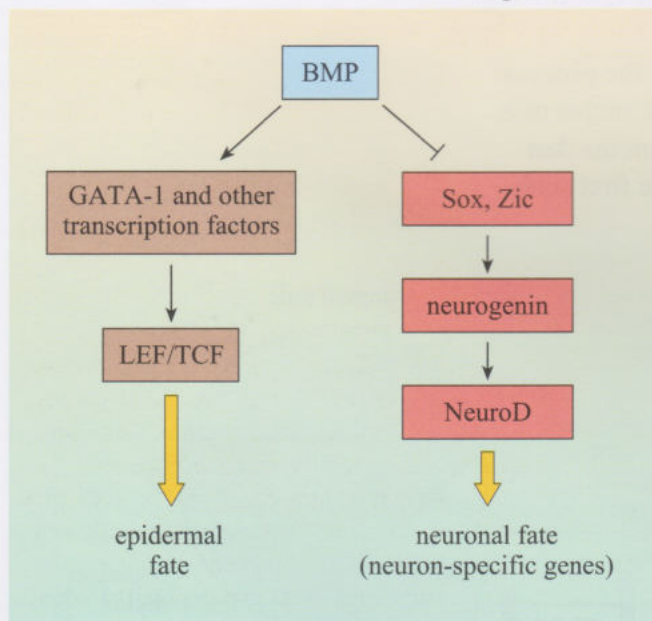


Figure 17.19 Downregulation of BMP signalling initiates a cascade of expression of transcription factors (shown in red boxes) that promote neuronal differentiation, and downregulates signalling initiated by the transcription factor GATA-1 (beige), which promotes epidermal differentiation. (You do not need to memorize the components of the epidermal pathway.)

The cells of the neurectoderm are often known as **neural precursors**, or **progenitors**; they are mitotic cells and give rise to the different neuronal types and also to glial cells, depending on the time and place of their ‘birth’ (i.e. withdrawal from the cell cycle) and the signals that they receive.

The genes responsible for the differentiation of neurons in vertebrates turn out to be homologous to the so-called **proneural genes** identified in *Drosophila*. Two transcription factors that play key roles in determination of neural fate are neurogenin and NeuroD (Figure 17.19). Contact-mediated signalling, such as that involving Notch and Delta (Table 17.1), plays an essential role in the regulation of neurogenin and NeuroD expression, as we shall see next.

In order to consider these events more closely, we return to the *Xenopus* embryo. The neural plate initially consists of self-renewing neuroepithelial stem cells that all have the potential to become neurons, but not all of them do so. The first neurons begin to differentiate in the neural plate. In mammals, although similar molecules and mechanisms are involved, neuronal differentiation begins after closure of the neural tube. What determines which cells will become neurons?

In the *Xenopus* neural plate, genes of the *Sox* and *Zic* families (Figure 17.19) promote expression of neurogenin by all the cells, which also express the cell surface protein Delta and its receptor Notch. Binding of Delta to Notch inhibits neurogenin expression in the Notch-expressing cells. Initially, the interaction of Delta and Notch between adjacent cells causes mutual inhibition of neurogenin expression, as shown in Figure 17.20a.

Although all the cells express Notch and Delta, the levels of expression may vary slightly from cell to cell. When a cell happens to produce slightly more Delta, signalling via Notch receptors in adjacent cells is promoted, leading to a further downregulation of neurogenin expression in these cells (Figure 17.20b). Since neurogenin also stimulates Delta expression, the decrease in neurogenin levels in turn leads to downregulation of Delta in those cells. Hence the reciprocal signalling to Notch in the first cell is reduced, leading to further increase in neurogenin and therefore Delta expression by that cell. Neurogenin also promotes expression of NeuroD, a transcription factor that commits cells to the neural fate and is necessary for neuronal differentiation (Figure 17.19). Hence the original cell in which Delta expression is increased goes on to develop as a neuron, while inhibiting neuronal differentiation of the adjacent neural precursor cells.

Given the complexity of the nervous system, it is perhaps not surprising that the regulation of neuronal differentiation is also complex. In addition to genes that promote neuronal (and glial cell) differentiation, genes that repress neural differentiation, and so maintain a pool of self-renewing neural stem cells have also recently been identified as essential in the patterning and differentiation of the nervous system.

The expression of proneural genes initiates a program of neural development, which involves the action of transcription factors that *upregulate* the expression of many neuron-specific genes, such as those encoding neurotransmitter receptors and neuron-specific cytoskeletal proteins. However, gene repression is also important in differentiation. Many neuron-specific genes are *silenced* in non-neural tissues. An example of how this occurs is by the action of repressor elements which silence transcription of neuron-specific genes by recruiting co-repressors, including histone deacetylases.

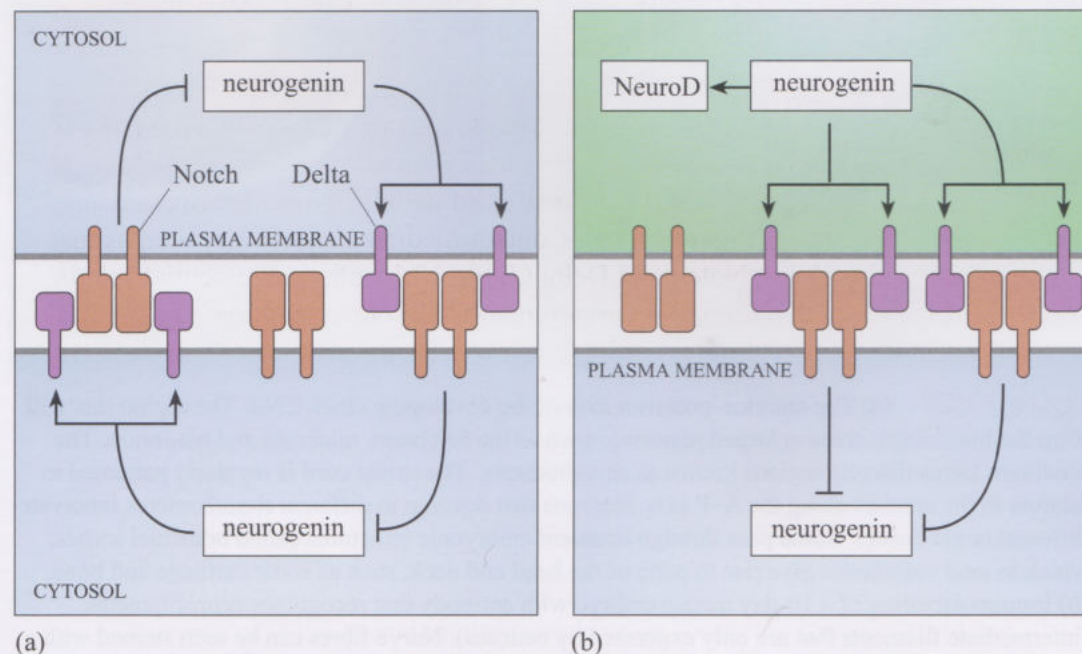


Figure 17.20

The role of Notch and Delta in neuronal differentiation. (a) Initially, adjacent cells both express Delta and Notch, and there is reciprocal signalling between the cells. Activation of Notch by Delta inhibits neurogenin expression, while neurogenin stimulates Delta expression. (b) When one cell (at the top) produces more Delta, Notch signalling in its neighbour (lower cell) is increased, leading to further downregulation of neurogenin expression by that cell, and also to reduced expression of Delta. This leads to increased neurogenin expression by the top cell, which in turn leads to the expression of more Delta and the transcription factor NeuroD, which commits the cell to a neuronal fate.

17.3.3 Hox genes and regional specification in the vertebrate nervous system

The vertebrate nervous system consists of the different regions of the brain and spinal cord (collectively known as the central nervous system, or CNS) and the peripheral nervous system. The neurons that develop in these different regions of the nervous system have different properties (they may utilize different neurotransmitters, for example) and crucially, they make specific connections with their target cells. The development of this neuronal circuitry therefore involves regulation of the differentiation of groups of cells within particular regions, as well as that of individual cells.

The regional differences evident along the anterior–posterior axis of the CNS become apparent soon after neurulation, as the most anterior region enlarges to form the forebrain, midbrain and hindbrain, as shown for the chick embryo in Figure 17.21. (Note that the early development of the mammalian CNS is similar.) Posterior to the hindbrain, the neural tube develops into the spinal cord, which has a regular pattern of organization that is determined in part by the somites.

Note that the hindbrain is organized into regular distinct regions, known as **rhombomeres**. The cells in different rhombomeres go on to differentiate into neurons that will innervate different targets (such as different muscles of the face). The specificity of this innervation is crucial for the neural circuits to function correctly.

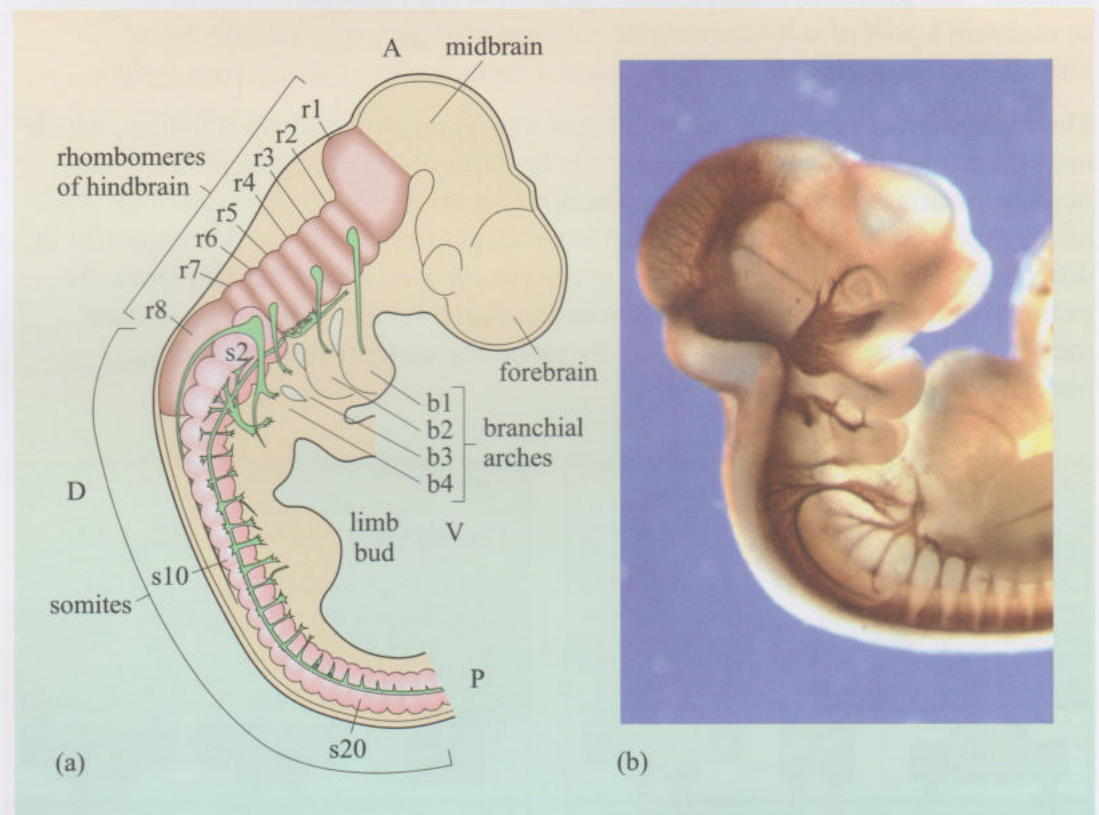


Figure 17.21 (a) The anterior–posterior axis of the developing chick CNS. The region that will form the brain forms three enlarged regions known as the forebrain, midbrain and hindbrain. The hindbrain forms discrete regions known as rhombomeres. The spinal cord is regularly patterned in relation to the somites along the A–P axis. Neurons that develop in different rhombomeres innervate different target tissues. Some pass through transient embryonic structures called branchial arches, which in land vertebrates give rise to parts of the head and neck, such as some cartilage and bone. (b) Immunolabelling of a 10-day mouse embryo with antibody that recognizes neurofilaments (intermediate filaments that are only expressed by neurons). Nerve fibres can be seen stained with a brown colour reaction product, extending from the rhombomeres. (Courtesy of Dr J. Golding)

This anterior–posterior specificity arises because rhombomeres form separate developmental regions with distinct boundaries within the developing hindbrain. Labelling and tracing of single cells has shown that before the appearance of the regular constrictions between the rhombomeres, cells can mix freely, but once the rhombomeres become apparent, although cells can move within a rhombomere, they cannot cross the boundaries between them. This restriction is thought to be due to the action of cell surface molecules known as **ephrins** (see Chapter 13, Section 13.2.2; and Table 17.1). Thus, this region of the CNS exhibits *segmentation*.

Segmentation of the body is a fundamental characteristic of many animals, including vertebrates, arthropods such as insects, millipedes and crustaceans, and annelid worms (e.g. earthworms). Our understanding of how the body plan of these animals is formed during development has come from seminal work on *Drosophila melanogaster*. Genetic analysis of mutant flies has revealed the existence of a crucial group of homeotic genes, that is genes that specify segmental identity of groups of cells, known as **Hox genes**. In common with other homeotic genes, Hox genes possess sequences known as **homeoboxes**, which are evolutionarily conserved between animals, and encode DNA binding domains known as homeodomains (Chapters 5 and 10; see also S204, Book 6, Chapter 5). Note that homeodomains are also found in other developmentally important regulatory genes, including some that play a role in the specification of neural fate (e.g. *Pax* genes, Section 17.3.4).

Different species possess related families of Hox genes, which occur as clusters in the genome, as shown in Figure 17.22. Within these clusters, the Hox genes are present in the same order along the chromosomes. There are two Hox gene clusters in the *Drosophila* genome; these are known as the Antennapedia and Bithorax complexes (Figure 17.22a). During evolution, gene duplication has given rise to clusters of increasing size, and duplication of clusters has also occurred, resulting in the occurrence of four clusters in vertebrates. These clusters are called *HoxA*, *HoxB*, *HoxC* and *HoxD* (Figure 17.22b). A striking characteristic of the Hox genes is that they are differentially expressed, in both a temporal and a spatial manner, in the order in which they are found along the chromosome. The body segments of the adult *Drosophila* result from earlier expression of specific Hox genes, as illustrated in the colour-coded Figure 17.22a. Spatial patterns of expression in the mouse embryo are shown in Figure 17.22b.

The names Antennapedia and Bithorax originate from the phenotype of flies that have mutations in these Hox gene clusters. For example, in Antennapedia mutants, legs develop instead of antennae.

Inspection of the colour-coded diagram of the mouse embryo in Figure 17.22b, shows that Hox genes are expressed in the hindbrain, spinal cord, mesoderm and their derivatives, including the limb buds. The forebrain and midbrain do not express Hox genes, but do express other regulatory genes that contain homeodomains. The endoderm and its derivatives also do not express Hox genes, but their development depends upon close interactions with mesodermal tissues that do.

The pattern of expression of the Hox genes is more complex than shown in Figure 17.22, in three ways. First, not all the cells in a particular region express Hox genes; second, some Hox genes are only expressed transiently during development; and finally, although the anterior boundary of expression of the Hox genes is clearly defined by a high level of expression, the posterior boundaries extend a considerable distance, and tend to be more diffuse, having lower levels of expression. This feature is shown schematically for the mouse mesoderm in Figure 17.23.

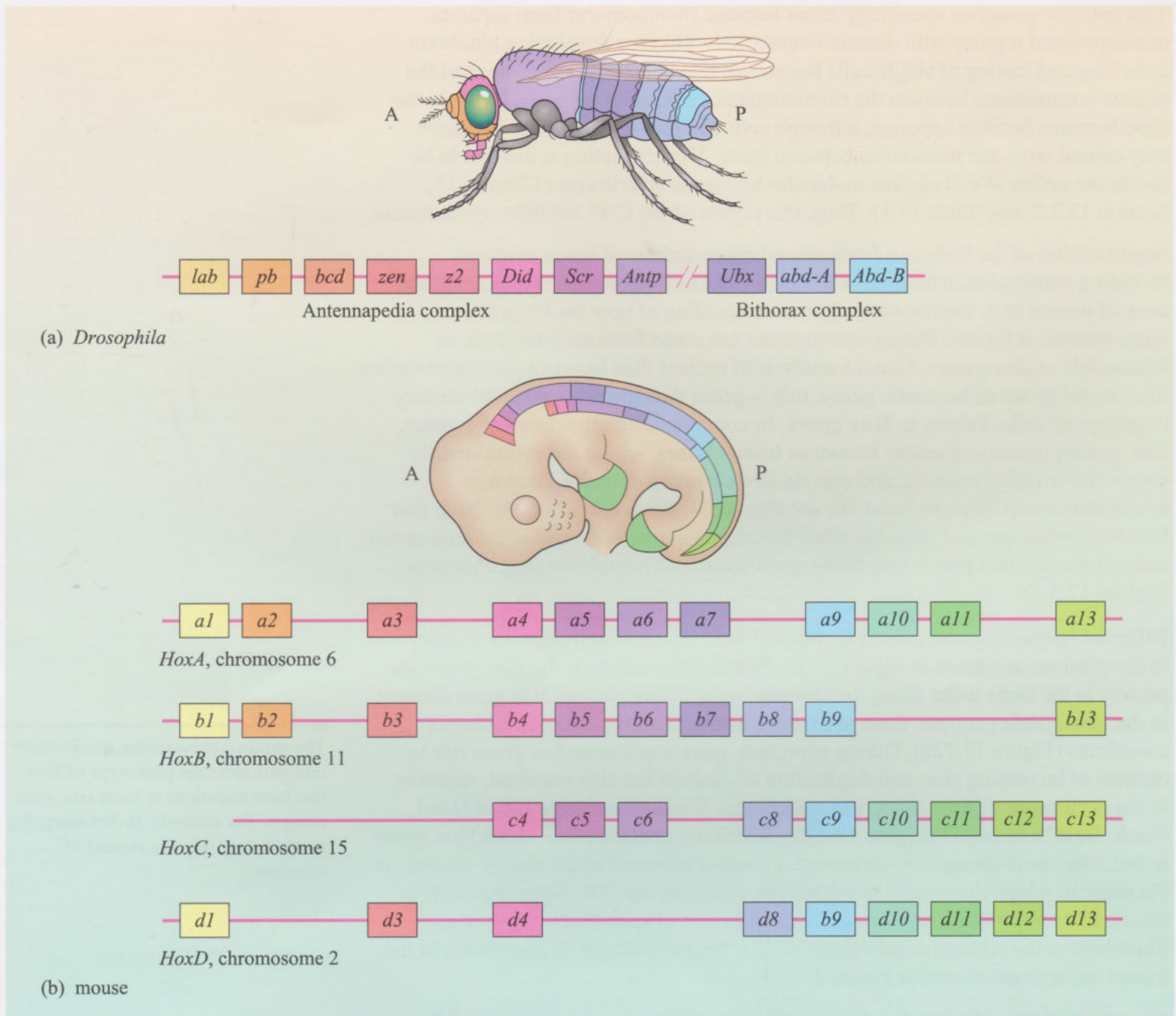


Figure 17.22

Hox gene complexes in (a) *Drosophila* and (b) mouse. The Hox genes involved in the development of the parts of the adult fly, and in the developing spinal cord, mesoderm and limb buds of the mouse embryo are shown colour-coded.

- ☐ From what you have learnt about the control of gene expression, suggest how the pattern of Hox gene expression (shown in Figures 17.22 and 17.23) provides positional information.
- ☒ Cells in a particular region will be exposed to different combinations and also different levels of the transcription factors encoded by the Hox genes.
- ☐ What is the name given to this type of regulatory mechanism?
- ☒ Combinatorial control.

The combination and levels of the transcription factors encoded by the Hox genes in cells in different regions along the anterior–posterior axis therefore provide these cells with different positional information, in the form of a particular pattern of regulatory gene expression.

**Figure 17.23**

Expression of Hox genes along the anterior–posterior axis of the mouse mesoderm, which will give rise to the different vertebral regions surrounding the spinal cord. For example, *Hox a6* is not expressed in the cervical region, but is expressed at its highest levels in the thoracic region with much lower levels of expression in the more posterior vertebral regions. Note that there are sharp boundaries of expression anteriorly, but diffuse boundaries posteriorly. Expression has been represented as a colour scale, from yellow (low) through orange (moderate) to red (high) levels. Regions with no expression lie outside the colour-scale bands.

The Hox gene family is found in almost all animals and is thus evolutionarily ancient. It is perhaps one of the best examples of how the mechanisms that regulate animal development are highly conserved.

The evidence for the importance of Hox genes in the determination of segmental identity in *Drosophila* is unequivocal and we do not describe it here. But what is the evidence for the roles of these genes in vertebrate development, particularly that of the nervous system? Study of transgenic animals has provided much information, and shows that the Hox genes play an essential role in regulation of the identity of neurons in the different regions of the hindbrain. For example, deletion of the *Hox b1* gene, which is normally highly expressed in rhombomere 4, results in a change in the fate of the cells such that they give rise to neurons that innervate different target tissues.

So, Hox genes play a role in the regional specification of the vertebrate nervous system, and in the determination of neuronal identity, as well as acting on mesodermal derivatives. What drives the differential expression of the Hox genes in the first place? The answer is by no means certain, although in vertebrates, expression of Hox genes begins during gastrulation. The Hox genes that are expressed in the anterior mesoderm and neural tube are the first ones to be expressed, followed by progressively more posteriorly expressed Hox genes. This has led to the suggestion that a signal from the chick equivalent of the organizer may be responsible. One candidate for this signal is the vitamin A derivative, **retinoic acid**, which is a small hydrophobic molecule that passes readily through cell membranes and activates intracellular receptors (Section 13.2.4). Retinoic acid has been shown to affect Hox gene expression in a dose-dependent manner, which is why high doses of vitamin A can have teratogenic effects, by disrupting the normal pattern of Hox gene expression in the developing limb.

A teratogen is a substance that causes developmental abnormalities.

Retinoic acid is produced by mesoderm near to the organizer, such that a dorso-ventral gradient is set up in the dorsal mesoderm of the late gastrula. Since presumptive neurectodermal cells pass close to this source during gastrulation, one proposed model is that differential exposure to retinoic acid promotes differential expression of Hox genes. Indeed, it has been proposed that the neurectoderm is exposed to different signals from different regions of the mesoderm (Figure 17.24). Like many other aspects of the regulation of differentiation, however, this hypothesis has not yet been confirmed and other molecules, such as FGF and Wnt family members (Table 17.1) may well be involved.

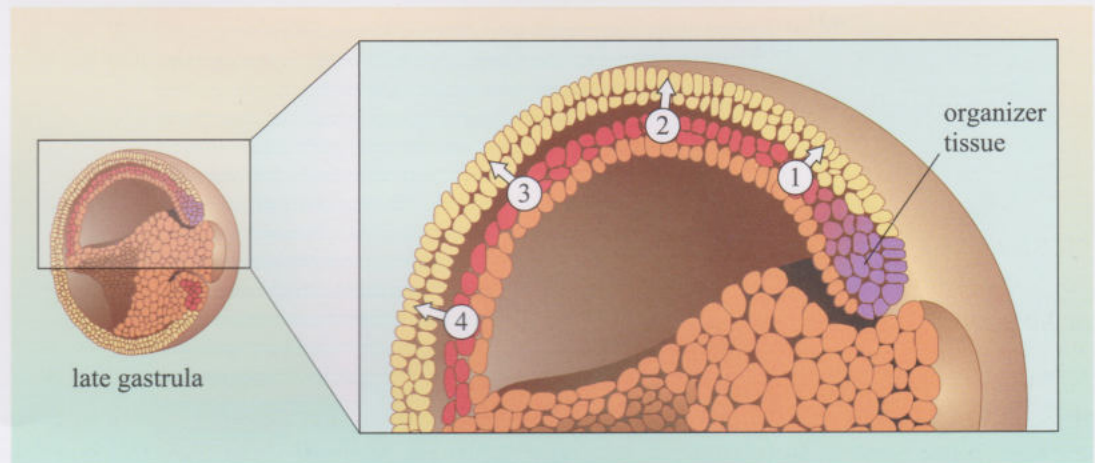


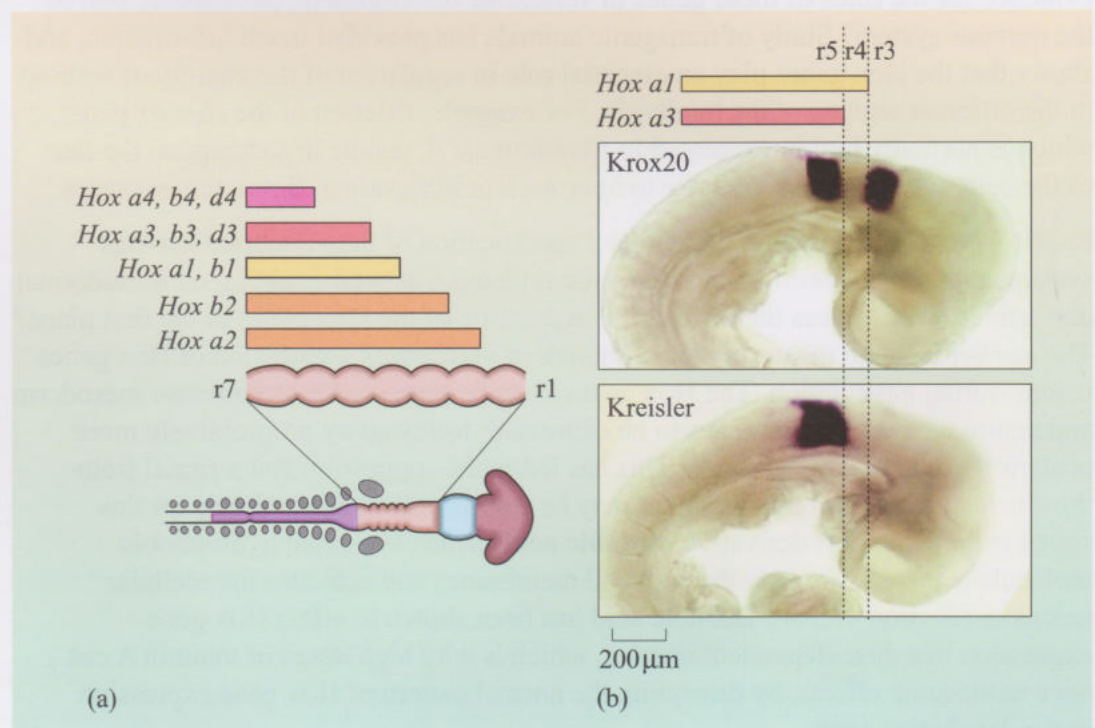
Figure 17.24

A model for the regulation of neural axis specification in *Xenopus*. Signals from different regions of the mesoderm, (e.g. 1, 2, 3 and 4) to the overlying neurectoderm may be different.

Whatever the initial signal, transcription factors other than those encoded by Hox genes, are also likely to be involved. For example, in the hindbrain, the pattern of expression of Hox genes is dependent upon localized expression of the transcription factors Krox and Kreisler, which are shown in Figure 17.25. Disruption of Krox results in loss of rhombomeres 3 and 5, while Kreisler disruption leads to loss of rhombomere 5 and abnormal formation of rhombomeres 3 and 6.

Figure 17.25

(a) shows the expression of various Hox genes in the developing chick hindbrain. Different patterns of expression specify the properties of the cells in different rhombomeres. For example, as indicated in (b), *Hox a1* is expressed in rhombomere 4 and 5, and more posterior regions, but not in more anterior regions. *Hox a3* is expressed in rhombomere 5 and more posterior regions. (b) shows the regulatory gene Krox 20 is expressed in rhombomeres 3 and 5, while Kreisler is expressed in rhombomere 5. (Data from Borday *et al.*, 2003)



17.3.4 Specification of different types of neurons involves diffusible signals

The nervous system is composed of many different types of neurons. How are these different neuron types specified? By now, you should be able to suggest some general mechanisms.

- How could different neuronal subtypes be specified?
- Different neuronal precursors will possess specific positional information – for example, by expression of a particular complement of Hox genes. They will receive a unique combination of signals from adjacent cells and diffusible signalling molecules. They will therefore express specific combinations of transcriptional regulators.

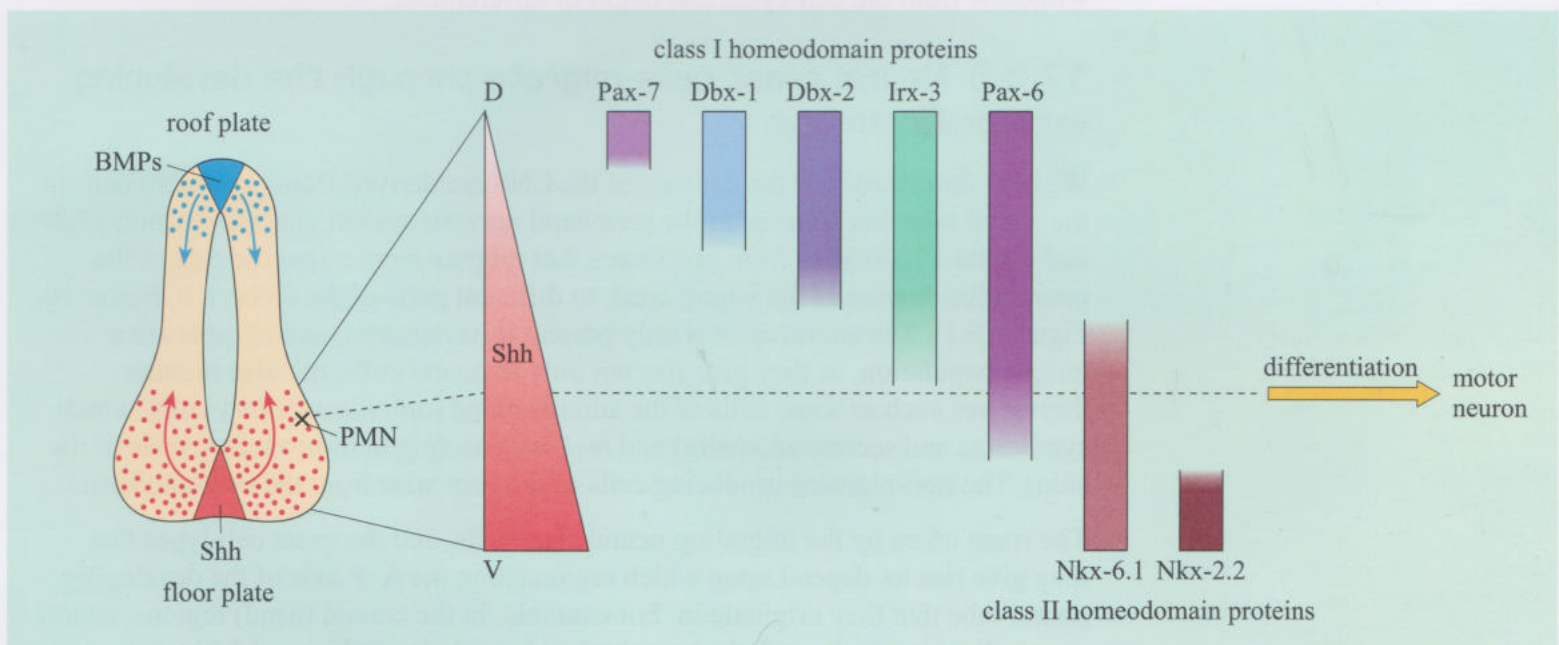
Here we shall consider the example of the specification of **motor** neurons, i.e. neurons that regulate movement by inducing contraction of skeletal muscles. Motor neurons are only one of several types of neuron that are present in the ventral part of the vertebrate spinal cord (other types include interneurons, which make connections with other neurons).

After neural tube closure, two nearby regions secrete signalling proteins that specify the fate of the neural precursors in the developing spinal cord. These are the roof plate and the floor plate, shown in Figure 17.26. BMPs are secreted by cells in the roof plate, whereas **sonic hedgehog (Shh)** (see Table 17.1) is secreted by cells in the floor plate and the notochord. Gradients of these proteins specify the different types of neuron in the spinal cord. Here we show how the gradient in Shh can specify different populations of ventral spinal neurons, by stimulating or inhibiting expression of different homeodomain genes, as shown in Figure 17.26.

The fate of neural precursors in the spinal cord is therefore determined by the combination of transcription factors that they express, which is, in turn determined by their exposure to different levels of Shh and BMPs.

Figure 17.26

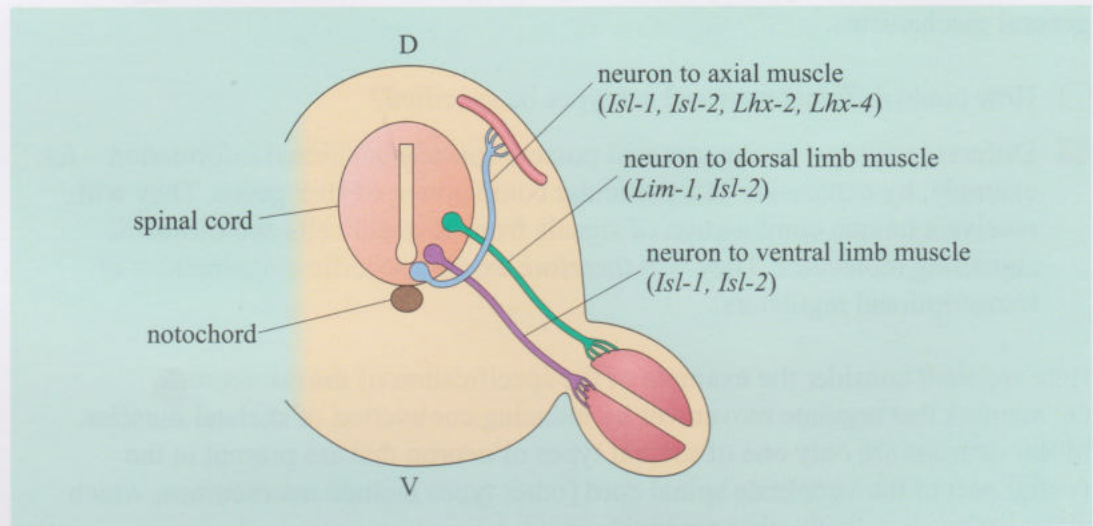
Gradients of BMPs and sonic hedgehog (Shh) specify different neuronal types in the developing vertebrate spinal cord. BMPs are produced by the cells of the roof plate; Shh is produced by cells of the floor plate and the notochord (not shown). Subtypes of spinal cord neurons are generated as a result of the gradient of Shh along the ventral–dorsal axis of the spinal cord. The effects of Shh are concentration-dependent, as shown. The neural precursor cells are stimulated to express different combinations of regulatory genes by exposure to different levels of Shh. For example, precursors of motor neurons (PMN), are present at the position along the developing spinal cord indicated by a cross. They are therefore exposed to moderate levels of Shh, which inhibit expression of all the class I homeodomain proteins shown, except Pax-6, but stimulate expression of the class II homeodomain protein, Nkx-6.1. The PMN go on to differentiate into motor neurons. (Note that you do not need to remember the names of the homeodomain proteins.)



Motor neurons are not a single group of cells; even at a single position along the length of the spinal cord, there are different types of motor neuron, which innervate different skeletal muscles. These different types of motor neuron express different combinations of further regulatory genes, as illustrated in Figure 17.27.

Figure 17.27

Simplified representation of some subtypes of motor neuron in the spinal cord of the developing chick. The neurons innervate different target muscles, and express different regulatory genes (in brackets). (You do not need to remember the names of these genes.)



- ☐ From Figure 17.27, apart from their projection to different skeletal muscles, what distinguishes these three types of motor neuron?
- ☒ Their position within the spinal cord along the D–V axis, and the combination of regulatory genes that they express.

What regulates the expression of specific regulatory genes by different types of motor neuron? The regulatory genes expressed by different motor neurons are determined by the signals that the neural precursors receive by virtue of their position within the spinal cord, and the signals that they receive as their processes begin to grow out of the spinal cord into the developing embryo. There is also a temporal aspect to the differentiation of motor neurons; the signals received by developing motor neurons will depend upon the time at which the neural precursors withdraw from the cell cycle and begin to differentiate.

17.3.5 Neural crest cells migrate through the developing vertebrate embryo

We have described how the neurons of the CNS are derived from progenitor cells in the neural tube, but neurons of the peripheral nervous system ganglia (Section 17.2 and Figure 17.29) arise from precursors that migrate from a specific part of the neural tube, known as the neural crest, to different parts of the embryo (Chapter 16, Figure 16.1). The neural crest is only present in vertebrates, and its cells are a unique population, as they give rise not only to neural cells, but also to other derivatives such as some cells of the adrenal gland (*adrenomedullary* cells, which synthesize and secrete adrenalin) and *melanocytes* (pigment-producing cells of the skin). The non-pigment-producing cells of the skin arise from the *neurectoderm*.

The route taken by the migrating neural crest cells, and the exact cell types that they give rise to, depend upon which region along the A–P axis of the developing neural tube that they originate in. For example, in the cranial (head) regions, neural crest cells migrate through the branchial arches (shown in Figure 17.21) and give

rise not only to peripheral neurons but also to cranial cartilage. The differentiation of neural crest cells is likely to be in part specified by the anterior–posterior positional information received at their site of origin in the neural tube, and by signals that they encounter during migration and at their final destinations. Here we are going to discuss the migration of neural crest cells, but not their differentiation. Why does this population of cells undergo such extensive migration, and how is the process controlled so that they do not invade the entire embryo?

First we consider why the neural crest cells are able to migrate and what promotes their emigration from the neural tube; then we turn to examine the factors that determine the routes taken by the neural crest cells in the developing embryo.

The neural crest forms from cells located in lateral regions of the neural plate (i.e. the border of the neural plate and the non-neural ectoderm), which come together as the tube closes (Figure 17.28). Evidence suggests that specification of presumptive neural crest occurs at the neural plate stage, and that signalling proteins that you are already familiar with and which originate in adjacent non-neural ectoderm and underlying mesoderm are involved. BMP gradients, FGF and Wnt signalling have all been implicated, but their precise roles remain to be fully established, and it is likely that differences in the molecular mechanisms of neural crest cell specification may exist between vertebrate species (see Gammill and Bronner-Fraser, 2003).

The genes that are induced in the presumptive neural crest cells in response to the inductive signal also remain unknown. A bewildering number of different genes, mostly encoding transcription factors, have been found to be either up- or down-regulated in presumptive neural crest cells, and the picture is again complicated by differences between different vertebrate species. Nonetheless, several genes have emerged as strong contenders for the early markers of neural crest identity, and it is hypothesized that their products go on to regulate neural crest cell fate.

At the time of writing, two of the best-studied of these genes are *Slug* and the related gene *Snail*. *Slug* protein is expressed in chick pre-migratory neural crest cells and *Snail* is expressed in those of mice and fish (*Xenopus* neural crest expresses both *Slug* and *Snail*). The *Slug* promoter contains a LEF/TCF binding site.

- ☐ What is the implication of the observation that the *Slug* promoter has a LEF/TCF binding site?
- ☒ The LEF/TCF transcription factor is activated in response to Wnt signalling (Section 17.2.4). From this it can be implied that Wnt signalling plays a role in neural crest induction.

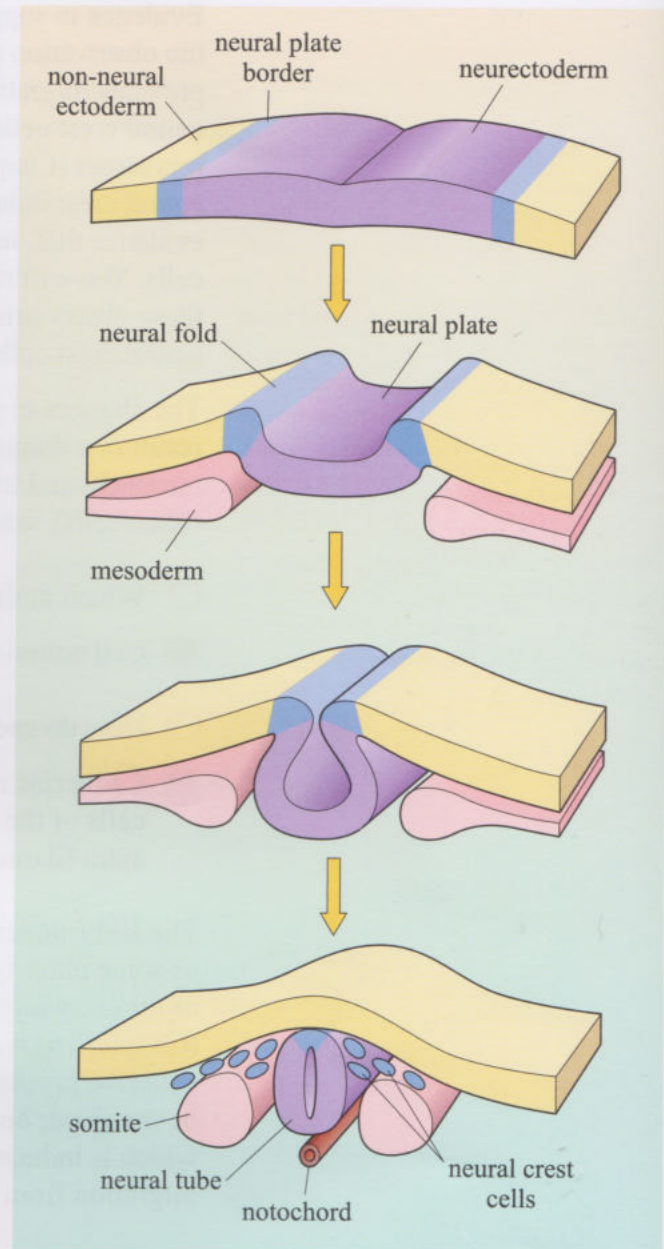


Figure 17.28 The presumptive neural crest develops at the border between the neural plate and the ectoderm. As neurulation begins, the presumptive crest is at the upper part of the neural folds, and comes together at the top of the newly forming neural tube. After closure of the neural tube, crest cells migrate away from the tube.

Evidence in support of a role for Slug in specification of neural crest cells includes the observation that inhibition of Slug expression by antisense oligonucleotides prevents migration, while overexpression of Slug increases the production of neural crest cells. Slug and Snail are zinc finger proteins that act as transcriptional repressors (Chapter 10). What are the downstream targets of Slug and Snail during neural crest induction? At present, they have not been identified, but there is evidence that, in culture, these proteins inhibit E-cadherin expression by epithelial cells. You will recall from Chapter 16 that cadherins are cell adhesion molecules, so these observations have implications for the adhesive properties of the presumptive neural crest cells (and therefore for their migratory abilities).

The changes in gene expression that occur in the presumptive neural crest cells result in a dramatic phenotypic change in the cells, known as an epithelial–mesenchymal transition (EMT; mesenchymal cells are loosely packed connective tissue cells), which, amongst other things, allows them to migrate.

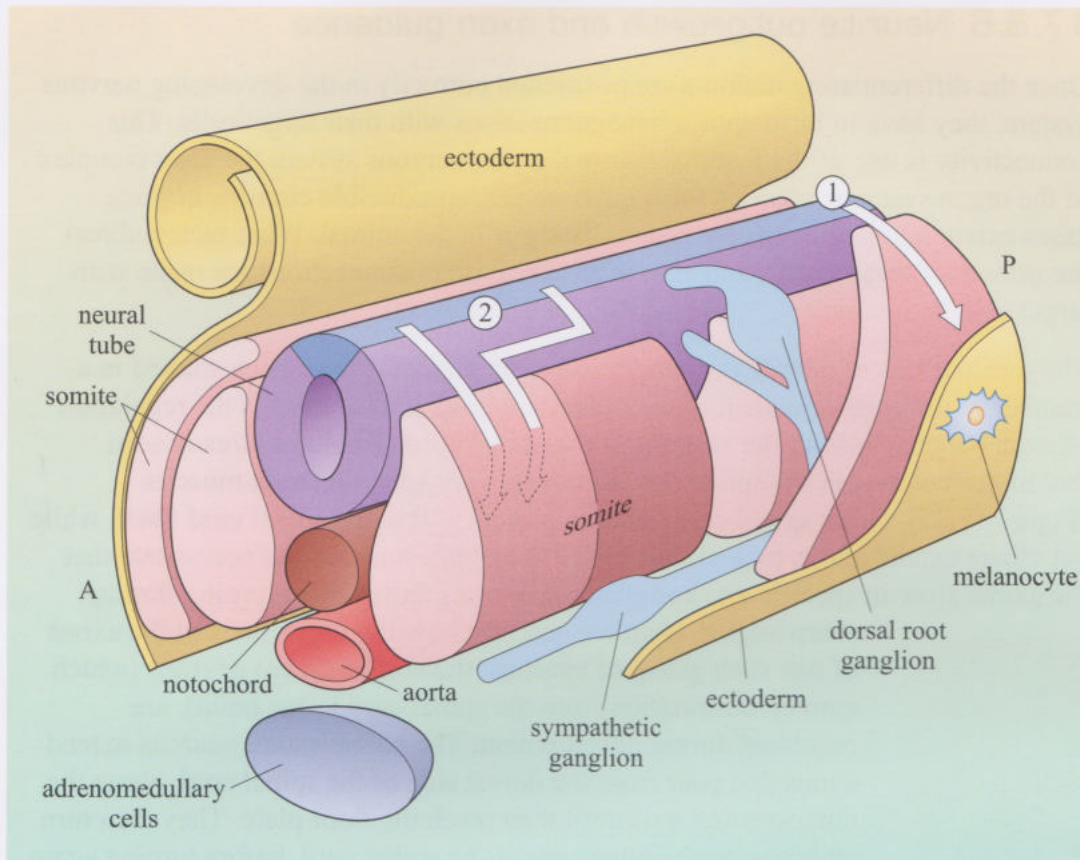
- ☐ Which molecules mediate the close contacts between adjacent epithelial cells?
- ☒ Cell adhesion molecules, such as E-cadherin (Chapter 16).
- ☐ How do cadherins mediate cell–cell interactions?
- ☒ Cadherins mediate Ca^{2+} -dependent homologous interactions between adjacent cells of the same type. Cadherins traverse the plasma membrane and link to actin filaments via the adaptor proteins, catenins.

The EMT undergone by the neural crest cells is accompanied by a downregulation of some other types of cadherin, including N-cadherin, but the upregulation of others, which are expressed by the migrating cells. Expression of N-CAM (Chapter 16) is also downregulated. The EMT thus results in a change in the adhesive properties of the neural crest cells. Additional molecules are also likely to be involved; one is RhoB, a member of the Rho family of GTPases (Chapter 16), which is induced in neural crest cells, and its inhibition prevents neural crest cell migration from the neural tube.

- ☐ What is the function of the Rho family of GTPases?
- ☒ They regulate changes in cell shape, by interaction with cytoskeleton-associated proteins (Chapter 16).

Having acquired a phenotype that allows them to move, the neural crest cells migrate along specific routes in the embryo. At this stage, as they have left the neural crest they are, strictly speaking, neural crest-derived cells. Classical experiments by Nicole Le Douarin and her co-workers, who used chick–quail interspecies grafts to follow the fate of transplanted cells, revealed much about the pathways taken by neural crest cells, and their fates when they reached their destinations. More recently, other types of labelling, such as labelling of individual neural crest cells by microinjection of fluorescent dyes, have been used to establish the fate of the cells that leave the neural crest.

A transverse view of the pathways of migration taken by neural crest-derived cells of the embryo was shown in Figure 16.1. A three-dimensional view, showing more details of the routes at this level, is given in Figure 17.29.

**Figure 17.29**

Routes taken by neural crest cells at the level of the somites in the middle region of the embryo. Route 1: neural crest cells migrate under the ectoderm, and will give rise to melanocytes of the skin. Route 2: neural crest cells migrate through the anterior part of the somites in a dorso-ventral direction, and will give rise to peripheral sensory (dorsal root) ganglia and sympathetic ganglia (the future sites of which are shown). Neural crest cells also give rise to the adrenomedullary cells, which make up the part of the adrenal gland that synthesizes adrenalin. The future sites of melanocytes, dorsal root and sympathetic ganglia and adrenomedullary cells in the adult organism are shown in light blue.)

In the middle region of the embryo, there are two pathways of neural crest cell migration. One route is between the somites and the ectoderm; the cells that follow this route give rise to melanocytes in the skin (route 1 in Figure 17.29). The other pathway is more ventral (route 2); some cells that follow this pathway pass through the somites, and give rise to ganglia of the peripheral nervous system and the cells of the adrenal medulla; others remain near the neural tube and form ganglia near the future spinal cord (dorsal root ganglia).

The ventral pathway is a very specific route; neural crest cells pass through only the anterior portion of each somite. Evidence indicates that repulsion is involved in this guidance and that properties of the somites themselves are important. In experiments where the anterior and posterior regions of somites are transposed, neural crest cells still migrate through what were the anterior regions. Ephrin and Eph receptors (Table 17.1) are likely to mediate at least part of this guidance; neural crest cells express Eph receptors, while cells in the posterior regions of the somites express ligands of the ephrin family.

The extracellular matrix components of the pathways are also involved in determining the migration of neural crest-derived cells. Many extracellular matrix molecules have been identified, by immunohistochemical techniques, along the pathways taken by migrating neural crest-derived cells. For example, blockade of the integrin $\beta 1$ subunit *in vivo* inhibits migration of neural crest cells in some regions, indicating that laminin and/or fibronectin are involved (Chapter 16) and *in vitro*, neural crest cells migrate on fibronectin, laminin and collagen substrata.

Once the neural crest-derived cells reach their destinations, they undergo further changes, and begin to differentiate into different types of peripheral neuron, glial cells or other cell types.

17.3.6 Neurite outgrowth and axon guidance

Once the differentiating neurons are positioned correctly in the developing nervous system, they have to form appropriate connections with their target cells. This connectivity is one of the features that makes the nervous system the most complex of the organ systems; neurons form intricate yet reproducible circuits, in some cases extending their axons over long distances in the animal. What factors direct the growth of these axons, and ensure that the correct connections are made with target cells?

Neurite outgrowth refers to the growth of processes from neuronal cell bodies in *any* direction, whereas *axon guidance* refers to the specific direction taken by a unique process, the axon.

The mechanisms of neurite outgrowth and axon guidance have been studied in a number of different organisms. One of the best-studied examples is the regulation of axon outgrowth from the neurons of the spinal cord. You have already seen that motor neurons in the spinal cord innervate very specific target muscles (Figure 17.27). Other spinal cord neurons project within the spinal cord itself, while yet others extend axons to the brain. Often these different circuits necessitate that the axons grow in specific and complex trajectories that involve turning through

sharp angles. Here we describe how the projections of the axons of one such group of neurons, the **commissural** neurons (which convey information from the spinal cord to the brain), are regulated during development. The commissural neurons extend axons that pass from the dorsal side of the spinal cord, along the dorso-ventral axis until they reach the floor plate. They then turn and cross to the other side of the spinal cord, before turning again to project in an anterior direction to reach the brain (Figure 17.30).

The process of axon guidance is a specialized example of directional outgrowth through the extracellular matrix, and involves a number of mechanisms, cellular structures and molecules that were described in Chapter 16.

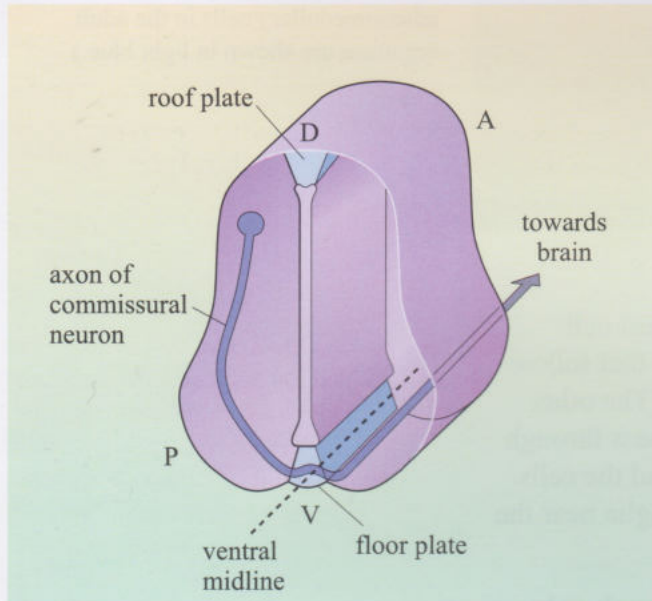


Figure 17.30 The axons of commissural neurons in the spinal cord project ventrally, then cross the ventral midline, and then project in an anterior direction to the brain.

- ☐ What are the structures that are extended at the leading edge of moving cells?
- ☒ Pseudopodia, lamellipodia or filopodia, depending upon their appearance.

Neurons have a specialized structure known as the **growth cone**, which is at the tip of the growing axon and both moves and acts as a sensor of environmental signals. The growth cone in some ways resembles the leading edge of a moving fibroblast; it continually extends and retracts filopodia, between which are ruffle-like lamellipodia (Figure 17.31).

The direction taken by the growth cone of a neuron is determined by two types of signal: molecules that the filopodia contact, and diffusible signals. Each of these may be attractive or repulsive, as illustrated in Figure 17.32.

Attractive stimuli maintain the filopodia, whereas repulsive stimuli cause them to collapse. Thus sources of chemoattractive or chemorepulsive signals can direct the growth of the axon.

The mechanism by which the axons of commissural neurons are guided across the ventral midline to the opposite side of the developing spinal cord involves the diffusible molecules netrin and semaphorin and contact-mediated signalling between the Slit ligand and its receptor, Robo, which you encountered in Chapter 16.

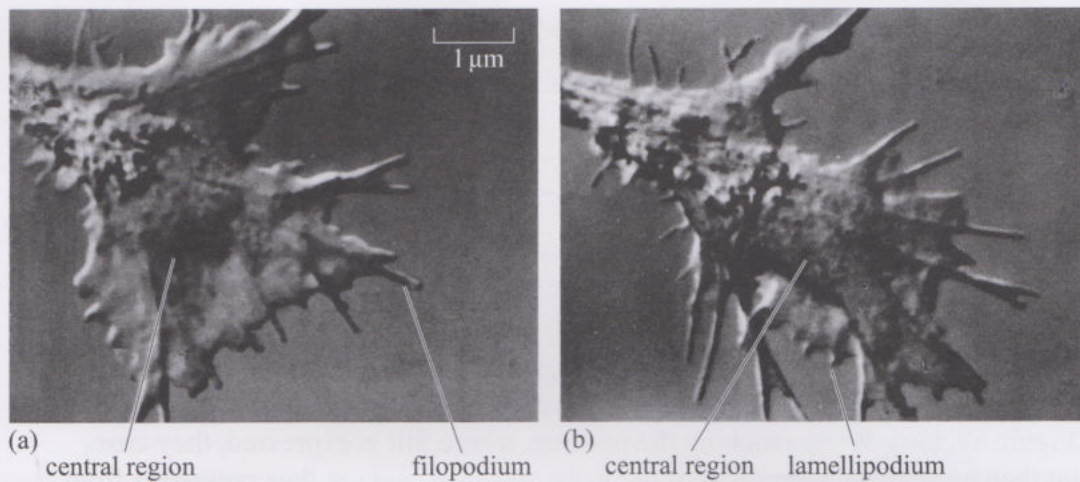


Figure 17.31 Video images of a growth cone from a neuron in the giant sea-slug (*Aplysia*); image (b) was taken seven minutes after (a).

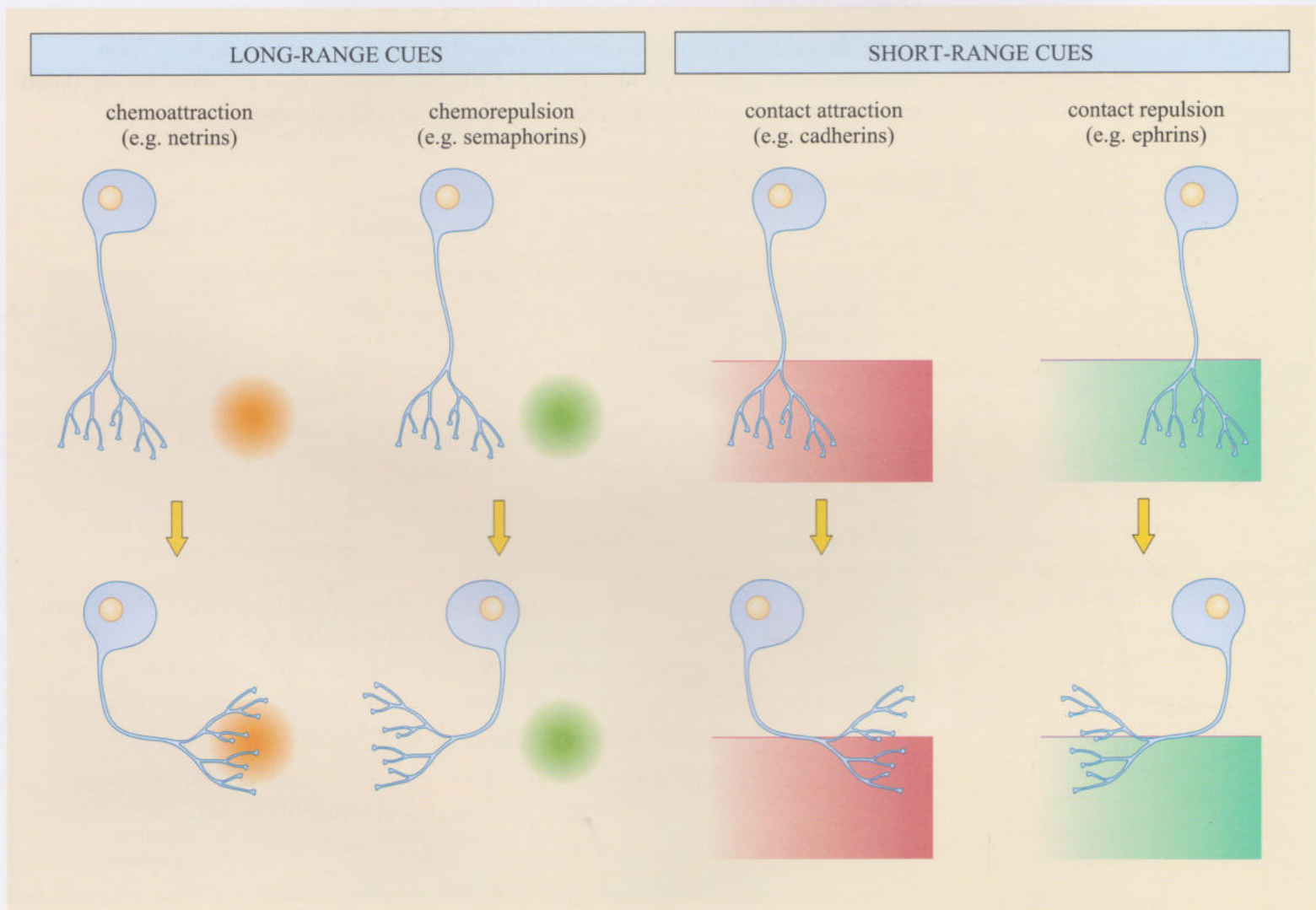


Figure 17.32 Axon guidance is mediated by contact and diffusible signals that act as chemoattractants or chemorepellents. Long-range cues involve diffusible signals; attractants include netrins; repellents include semaphorins. Short-range cues involve contact-mediated signalling and include attractant signals, such as cadherins and repellent signals, such as ephrins. The cellular response to netrin may be either attraction (as shown here) or repulsion, depending upon the molecular characteristics of the responding axon.

- ☐ What is the nature of the interaction between cells that express Slit and Robo?
- ☒ Slit ligand inhibits the migration of cells that express the appropriate Robo receptor (Section 16.6.3).
- ☐ What is the mechanism of this inhibitory interaction?
- ☒ The mechanism involves interference with signalling from G protein-coupled receptors via chemotactic signalling molecules (Section 16.6.3).

The growing axons are attracted by netrin, which diffuses from the floor plate at the ventral midline, because the growth cones express the netrin receptor (Figure 17.33a). When reaching the midline, where Slit is expressed, they cross but then upregulate expression of the Robo receptor, and are thus prevented from crossing back over the midline. The axons also upregulate expression of receptors for semaphorin, which is expressed by the cells of the wall of the neural tube. Hence the axons grow between the two repulsive signals, towards the brain (Figure 17.33b).

Once the growing axons reach the tissues that they will innervate, they form synapses with target cells (Section 13.1.1). This process also involves the regulated expression of specific proteins, but we do not describe it here.

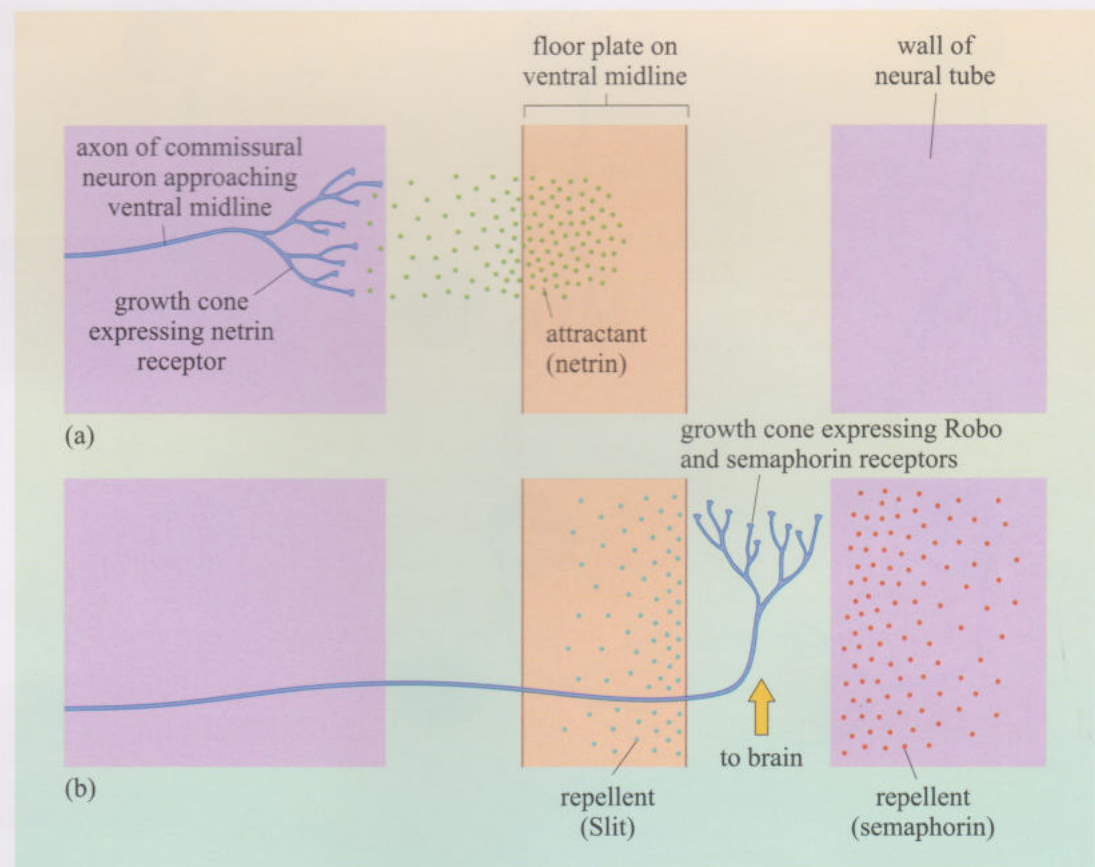


Figure 17.33 Guidance of axons of commissural neurons across the ventral midline. (a) The growing axons are attracted to the ventral midline by the secreted attractant protein, netrin. (b) Once they cross the ventral midline, they upregulate expression of Robo, and hence they are then repelled by Slit, which is also produced at the ventral midline. Another repellent, semaphorin, is produced by cells in the wall of the developing neural tube, hence the growing axons turn and grow between the two repellent signals, towards the brain.

Although Slit is usually expressed on the cell surface, it may sometimes be secreted.

Summary of Section 17.3

- 1 Development of the cells of the nervous system involves a series of events, including neural induction, neurulation, patterning of the central nervous system, neuronal differentiation, migration of neural crest cells and axon guidance.
- 2 In amphibians, neural induction involves inhibition of the action of BMP and Wnt proteins, by proteins that include Noggin and Frzb secreted by cells of the organizer.
- 3 Inhibition of BMP signalling in the presumptive neurectodermal cells results in downregulation of the transcription factor GATA-1 which drives epidermal differentiation, and activation of neural transcription factors, including neurogenin and NeuroD.
- 4 Increased expression of the cell surface signalling protein Delta results in increased activation of its receptor, Notch, and also of neurogenin in adjacent cells. This upregulation results in reduced reciprocal signalling from the adjacent cell, which stimulates increased expression of neurogenin in the first cell, which in turn promotes expression of the transcription factor NeuroD, and subsequent neuronal differentiation.
- 5 A dorso-ventral gradient in Hox gene expression along the developing nervous system provides positional information, which results in the formation of different regions along the anterior-posterior axis of the brain and spinal cord.
- 6 Different types of neuron are specified by expression of different regulatory genes, induced by exposure to different levels of signalling molecules, such as Shh and BMPs.
- 7 Neural crest cells give rise to a variety of cell types, including peripheral neurons and melanocytes. They arise from the neural tube, and undergo a transition from an epithelial to a mesenchymal state, which involves a change in expression of cadherins and other adhesion molecules. Neural crest cells migrate through the developing embryo along routes that are determined by cell surface molecules, ephrins, and by components of the extracellular matrix.
- 8 The projections of growing axons are determined by guidance cues; both diffusible and contact-mediated signalling are involved. Proteins in the environment of the growing axon are detected by receptors expressed on the growth cone. Axons of commissural neurons, which cross the developing spinal cord, are first attracted by netrins, and then their onwards trajectory is determined by repulsive signals from the proteins Slit and semaphorin.

17.4 Differentiation and plasticity in mature tissues

So far we have focused on differentiation that occurs during development, and we have perhaps viewed differentiation rather as if it were the final event in the life of a cell. In many tissues, however, differentiation continues throughout life, and there are also circumstances in which the properties of differentiated cells may change; this phenomenon is known as **plasticity**. In this section, we consider some examples of these processes in adult tissues. Two other examples where the properties of cells in adult organisms change are during cell senescence and tumorigenesis; these processes are considered in Chapters 18 and 19 respectively.

17.4.1 Intestinal epithelial cells

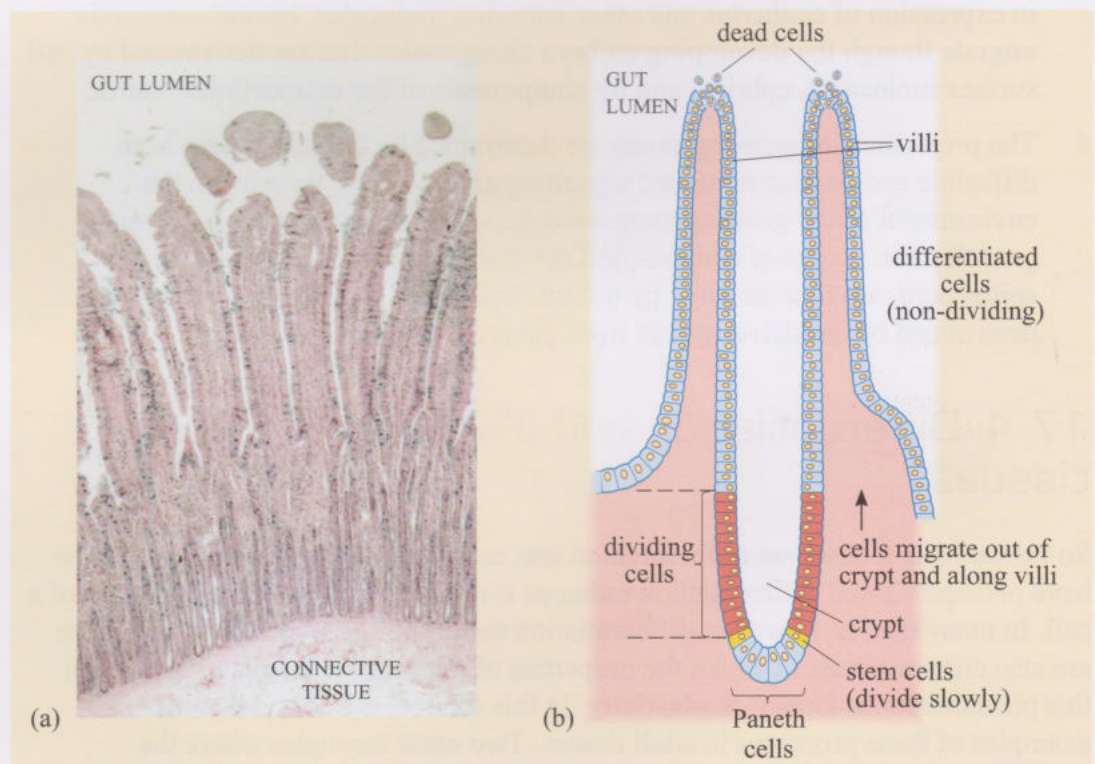
The intestine is lined by a specialized **epithelium** (a tissue made up of epithelial cells; epithelial tissues typically line body surfaces, such as the skin, gut and lungs). The intestinal epithelium is formed by a single layer of cells, which is shaped into finger-like projections, known as **villi**. Between the villi are the **crypts**, which project inwards into the connective tissues of the gut wall (Figure 17.34). There are four main types of differentiated epithelial cells in the mammalian small intestine; mucus-secreting goblet cells, absorptive enterocytes, enteroendocrine cells (which secrete local hormones), and Paneth cells, which are part of the innate immune system and secrete antimicrobial peptides into the lumen of the gut. The goblet, absorptive and enteroendocrine cells are located in the villi; the Paneth cells, however, are located at the very base of the crypts, as shown in Figure 17.34b.

Differentiated intestinal epithelial cells have a short lifespan (around two to three days in mice). Paneth cells die by apoptosis and their remains are phagocytosed by neighbouring cells. The goblet, absorptive and enteroendocrine cells, on the other hand, migrate to the tips of the villi, become senescent (Chapter 18) and are shed into the gut lumen. These cells are replaced from a pool of stem cells that have been found to lie in a specific position near the bases of the crypts (Figure 17.34). The stem cells give rise to progeny known as **transit cells** (and sometimes, *transit amplifying cells*), which continue to divide as they migrate along the crypts towards the villi and differentiate into goblet cells, enterocytes or enteroendocrine cells (Figure 17.35).

- ☐ What are the defining properties of stem cells?
- ☒ They are multipotent and self-renewing. They divide asymmetrically to give rise to daughter stem cells, which are also multipotent, and to daughter cells that will give rise to differentiated progeny (Section 17.1).

Figure 17.34

The life history of intestinal epithelial cells. (a) Light micrograph. (b) Schematic diagram showing the epithelial cells of the intestine. Stem cells in the crypts (finger-shaped indents in the gut wall) divide to give rise to other stem cells and to cells that migrate, divide and differentiate into specialized epithelial cells.



In the next section, we shall discuss some of the issues raised by this definition of stem cells. Here we consider the intestinal epithelial stem cells, and ask how they are studied, and what regulates their continued self-renewal and differentiation.

The proliferative properties of the intestinal stem cells and their progeny have been much studied in the small intestine of mice. Each crypt is effectively a unit, and contains some 250 cells, of which only 4–6 are stem cells. There are, on average, 16 cells around the circumference of each crypt. The stem cells reside at the 4th–5th positions above the base of the crypt (Figure 17.35). They do not always form a distinct layer, but may be intermingled with their daughter cells. The stem cells in the mouse small intestine have a cell cycle time of 24 hours; they divide throughout life, so it has been estimated that each stem cell divides some 1000 times during the lifespan of the mouse (mice live for about 2.5–3 years).

In humans, although the cell cycle time is longer, the number of times a stem cell divides in the small intestine is estimated to be between 5000 and 6000 (the average lifespan of a human is approximately 70 years; Chapter 18). Mutations that accumulate during repeated rounds of DNA replication are a major cause of tumours in humans (as you will see in Section 19.9.2), so it is remarkable that the incidence of tumour development is very low in the small-intestinal epithelium. In fact, there is a higher incidence of tumours of epithelial origin in the large intestine, where there are fewer stem cells, which undergo fewer divisions during the lifetime of the individual (Potten *et al.*, 2003).

Recent evidence indicates that intestinal stem cells have some special properties that protect them against DNA damage and also allow for their replacement if stem cells are destroyed – for example, by exposure to high levels of radiation. The first of these protective properties is that upon the second division of these cells, the newly synthesized DNA strands are passed to the cell that will become a member of the transit amplifying population.

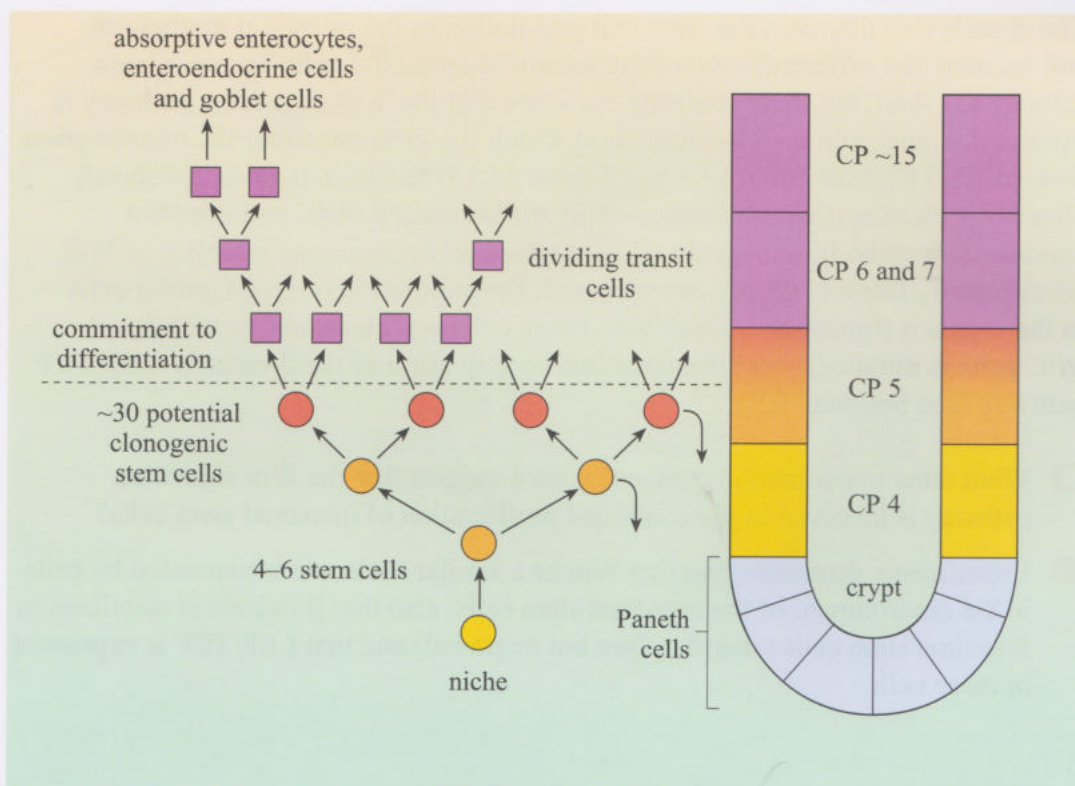


Figure 17.35
Cell lineage (left) and corresponding position in the crypt (right) in the intestinal epithelium of the mouse small intestine. Intestinal stem cells are located in a localized niche. Upon division, one daughter must leave the niche, becoming a potential clonogenic stem cell. These cells continue to divide, but as they move up the crypt, they become committed to differentiate. CP = cell position from the base of the crypt.

- ☐ What is the advantage of the selective sorting of newly synthesized DNA to the transit cell population rather than to the stem cells?
- ☒ Any errors made in the newly synthesized DNA will only pass to the daughter cell that is destined to differentiate and be shed from the epithelium a few days later, and not to the daughter stem cell.

Evidence for the selective segregation of template DNA has come from experiments in which DNA of intestinal stem cells of mice was labelled by repeated administration of radioactively labelled thymidine (tritiated, or ^3H -thymidine) during gut development (the first few weeks after birth). When these mice had become young adults (i.e. from about 3 months of age), their newly synthesized DNA was labelled using bromodeoxyuridine (BrdU, Box 9.1) and then identified using immunocytochemical techniques. These experiments revealed that over a period of time that involved several rounds of cell division, the thymidine label was retained in the intestinal stem cells, whereas the BrdU labelling was gradually lost (Potten *et al.*, 2003), indicating that the original, ^3H -thymidine-labelled DNA was selectively retained in the stem cells and their immediate progeny.

Another protective mechanism is that the DNA of the intestinal stem cell line is acutely sensitive to DNA damage. This property is demonstrated by the sensitivity of these cells to radiation damage; exposure to relatively low levels of radiation (0.01–0.05 Gy) stimulates p53-dependent apoptosis in the intestinal epithelial stem cells. When stem cells die in this way, neighbouring stem cells may divide symmetrically to produce two daughter stem cells, or one of their daughters may revert to a stem cell phenotype and so restore the stem cell population. It has been estimated that, at any time, there are some 30 such daughter cells that have the potential to revert to a true stem cell phenotype. These cells have been named **potential clonogenic stem cells** and represent an intermediate between the actual stem cells and the transit amplifying cells (see Figure 17.35).

The signals that maintain the stem cell population in the intestinal epithelium and regulate the differentiation of the intestinal epithelial cells have not been fully established, but there is strong evidence that the Wnt signalling pathway is involved. Transgenic knock-out mice in which the gene encoding the transcription factor LEF/TCF (see Table 17.1 and Figure 17.15) has been deleted die shortly after birth; their epithelium contains only differentiated cells, and does not contain stem cells. In transgenic mice in which an endogenous inhibitor of Wnt (Dickkopf-1, Table 17.1) is overexpressed, the population of proliferating cells in the crypts is diminished. Finally, in many colorectal tumours, in which the *APC* gene is mutated, there is a constitutive activation of the β -catenin–LEF/TCF pathway (see Section 19.9).

- ☐ What other experimental evidence would suggest that the Wnt signalling pathway is involved in the continued proliferation of intestinal stem cells?
- ☒ Experiments demonstrating that Wnt or a similar molecule is expressed by cells in the environment of the intestinal stem cells; also that β -catenin is stabilized in intestinal stem cells (and therefore not degraded) and that LEF/TCF is expressed in these cells.

Although the source of the Wnt signal has not yet been identified, recent evidence has demonstrated that β -catenin is indeed accumulated in the nucleus of stem cells in the adult intestine, and that the genes induced by β -catenin are expressed only by the proliferating cells, whereas differentiated cells do not express these genes.

17.4.2 Adult stem cells

The intestinal stem cells that we have just described are only one example of stem cells that are present in fully mature tissues of adult animals. Other examples include stem cells of the bone marrow (haemopoietic stem cells and mesenchymal stem cells), skeletal muscle stem cells (known as satellite cells) and neural stem cells. In recent years, the existence and properties of these cells have attracted much attention, as it has been proposed that they may provide a source of cells that could, following isolation and possibly after some manipulation in culture, provide a source of cells for cell replacement therapy in the treatment of certain diseases in humans.

But what are the normal properties of these cells? What drives their proliferation and differentiation, and what is their real potential?

There are problems with both the definition and the study of stem cells. True stem cells are **totipotent**; that is, they are able to give rise to any body cell type. Clearly, stem cells such as intestinal stem cells which reside in the gut normally only give rise to different types of intestinal epithelial cells, so are better described as multipotent.

- ☐ Why might it be inappropriate to define an intestinal stem cell as multipotent rather than totipotent?
- ☒ Although the intestinal stem cell *normally* only gives rise to intestinal epithelial cells, it may, under certain circumstances, such as when grafted to another site, have the ability to differentiate into *other* cell types.
- ☐ If such an experiment were performed and the intestinal stem cell gave rise to other differentiated cell types, what would be the problem with defining the intestinal stem cell as totipotent?
- ☒ The situation may be one that would never occur *in vivo*; the cell has been manipulated to behave in this way.

Adult stem cells are difficult to study, for several reasons. First, they are undifferentiated, and express few molecular markers that permit their ready identification. Second, they are, in effect, defined by their behaviour – the ability to divide asymmetrically so that they self-renew and give rise to progeny that can differentiate into a range of cell types. When stem cells are maintained in cell culture and treated with a variety of growth factors, they can be directed to differentiate along specific pathways into different cell types.

An example is the differentiation of neural stem cells, which can be isolated from several regions of the nervous system. When provided with a culture medium that contains epidermal growth factor (EGF, see Chapter 13) and fibroblast growth factor (FGF), these cells continue to divide, and do not differentiate. In the absence of a suitable culture substratum, they form clusters, known as **neurospheres**.

When EGF and FGF are removed, the cells differentiate into either neurons or glial cells, whereas addition of the cytokine ciliary neurotrophic factor (CNTF) promotes differentiation into glial cells.

Stem cells may be first isolated and then grafted into a different tissue or system in another experimental animal (Box 17.1). When transplanted to ectopic sites *in vivo*, some types of stem cells seem to be able to differentiate in a manner appropriate to the new site. This observation has led to the proposal that such cells could be used for cell replacement therapy. For example, evidence has been presented that neural stem cells may be able to give rise to skeletal muscle and blood, and that bone marrow stem cells may be able to give rise to neural cells, muscle cells and liver cells when transplanted into the appropriate tissue or system. Some of these results, however, remain controversial, and other evidence suggests that fusion between resident cells of the host tissue and transplanted stem cells may account for some of the reported observations.

More work has focused on true stem cells, the **embryonic stem (ES) cells**, which are totipotent and are derived from the inner cell mass of mammalian early embryos (Figure 17.36). When grown for indefinite periods in culture, ES cells can be manipulated to differentiate into all cell types by addition or removal of specific growth factors and/or hormones at critical times after isolation. In addition, ES cells isolated from one blastocyst can be reintroduced into other blastocysts and become incorporated into the host embryos.

The inner cell mass is a specific group of cells in the mammalian blastocyst, some of which go on to form the embryo (the rest form extra-embryonic structures).

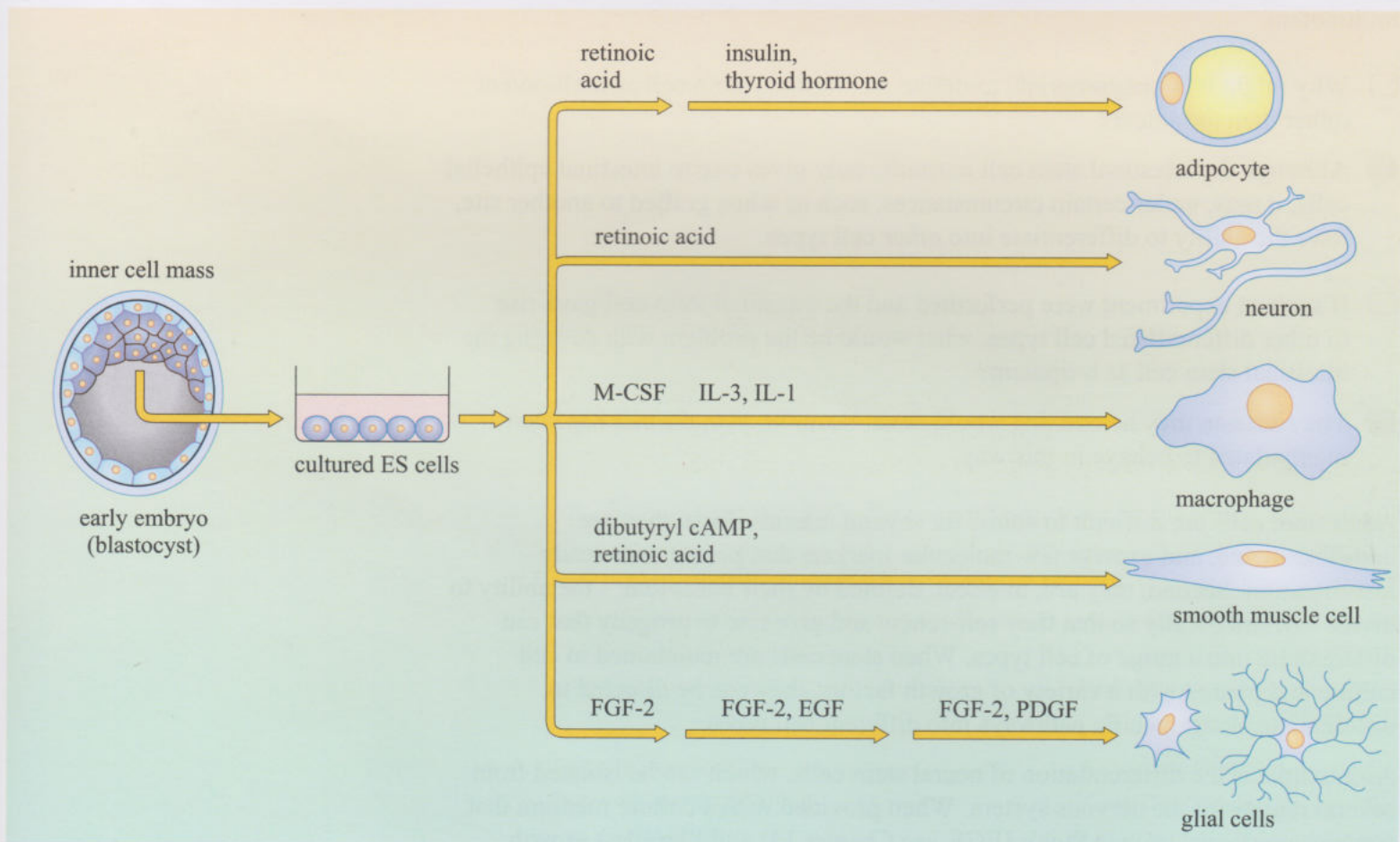


Figure 17.36 Embryonic stem (ES) cells and how they may be engineered in culture. Cells from the inner cell mass of the mammalian blastocyst may be grown *in vitro*, and can differentiate into the different cell types shown according to the factors that they are exposed to in culture. (M-CSF = macrophage colony-stimulating factor; IL = interleukin; FGF = fibroblast growth factor; EGF = epidermal growth factor; PDGF = platelet-derived growth factor.) Note that the factors involved in macrophage differentiation *in vivo* were shown in Figure 15.2.

17.4.3 Can cells de-differentiate?

Having seen something of how cells differentiate, we now turn to the question of whether they can de-differentiate. Clearly most cells, once differentiated, remain in their specialized state, but there are examples of plasticity, mostly in response to injury; for instance, the newt limb can regenerate following amputation.

Following amputation, epidermal cells migrate over the wound, and underlying cells de-differentiate and then re-differentiate into the different cell types (muscle, cartilage and connective tissue cells) that form a new limb. The nature of the cells that de-differentiate is unclear; evidence shows that they may be mesenchymal cells, but that muscle cells introduced into the environment can also **trans-differentiate** and form another differentiated cell type.

Some cells can also trans-differentiate in culture; an example is the change in phenotype of adrenomedullary cells, which are derivatives of the neural crest. Adrenomedullary cells have no processes (protrusions) and produce adrenalin when maintained in culture in the presence of steroid hormones (Section 13.2.4). When steroid hormones are removed from the culture medium and replaced with nerve growth factor (NGF, Chapter 14), these cells grow axons and begin to produce noradrenalin, properties of sympathetic neurons.

17.4.4 Cloning

Finally we turn to the issue of cloning. At the time of writing (Summer 2004), embryos from several species have been cloned, albeit with limited 'success' rates. Cloning involves the transplantation of the nucleus from a cell of one individual into the enucleated eggs (i.e. eggs from which the nuclei have been removed, usually by suction with a micropipette) of another individual. The nucleus could be from a fully differentiated somatic cell, such as the mammary epithelial cell nucleus that was used to clone Dolly the sheep, or from a partially differentiated cell. The nuclei used are normally transplanted into the enucleated egg during metaphase II of meiosis (S204, Book 2, Chapter 3).

The success of the technique relies on the **reprogramming** of the nucleus; that is, its patterns of gene activity must revert to those of the zygote, or very nearly. Perhaps remarkably, this reprogramming has been found to occur, albeit at a very low frequency. Most success has been achieved using *Xenopus*, and transplanting nuclei from donors at an early stage of development (see Table 17.2).

Clearly then, some reprogramming can occur, but what is the reason for this conspicuous lack of success in producing clones. The reasons may be different for *Xenopus* and mammals. In *Xenopus*, the main failure seems to occur at early stages after transplantation, and may be because there is insufficient time for replication of the donor DNA from somatic cell nuclei before the eggs begin cleavage (replication of chromosomes from somatic cells takes some 6 hours; cleavage takes place after 90 minutes).

In mammals, there may be a different problem, as the first cleavage division takes place 20 hours after fertilization, which should be sufficient for chromosome replication. The problem is likely to be due to a lack of reprogramming of silenced genes (Chapter 10; Section 10.5.3, p. 139) and/or removal of maternal spindle proteins needed for normal chromosome segregation during normal cleavage divisions.

Table 17.2 Success of nuclear transfers using nuclei from donors from different species and stages of development.*

Species	Donor cell source	Donor stage/age	% of nuclear transfers reaching muscular response stage†
<i>Xenopus laevis</i>	endoderm	muscular response stage	17–24
<i>Xenopus laevis</i>	endoderm	heart beat stage	9–13
<i>Xenopus laevis</i>	muscle	muscular response stage	2
<i>Xenopus laevis</i>	intestinal epithelium	early feeding tadpole	1–3
cow	inner cells mass	embryonic	0.6
primate	4–32-cell embryos	4–32-cell embryo	1.4
sheep	mammary	adult	0.4
mouse	cumulus (ovarian cells that surround the mammalian egg)	adult	2.8

* (Data from Gurdon *et al.*, 2003)

† The muscular response stage of the embryo is around the time of the tailbud stage (Figure 17.2).

Cloned embryos from both amphibians and mammals have been found to exhibit incorrect reprogramming; that is, the expression of zygotic genes is quantitatively abnormal, so that normal development cannot proceed. Interestingly, however, cells from such embryos can give rise to normal cells if grafted into host embryos, or can give rise to embryonic stem cell lines.

Summary of Section 17.4

- 1 Differentiation occurs in some tissues throughout life. An example is that of intestinal epithelial cells, which form from a pool of stem cells in the intestinal crypts.
- 2 Adult stem cells exist in many differentiated tissues. Evidence suggests that their potential *in vivo* and *in vitro* may be broader than previously thought.
- 3 Embryonic stem cells are derived from the inner cell mass of mammalian early embryos. They can be engineered in culture to produce specific cell types.
- 4 Some cells may be able to de-differentiate, after injury *in vivo*, or as a result of manipulation *in vitro*.
- 5 The transplantation of a nucleus from a fully or partially differentiated cell into an enucleated egg requires reprogramming of the genetic material.

Learning outcomes for Chapter 17

When you have studied this chapter, you should be able to:

- 17.1 Define and use each of the terms printed in **bold** in the text.
- 17.2 Explain, giving examples, how general mechanisms underlie the differentiation that occurs during development.
- 17.3 Briefly describe the main stages in the development of vertebrates, using *Xenopus laevis* as an example.
- 17.4 Explain the cellular processes involved in the main stages in the development of the vertebrate nervous system.
- 17.5 Compare, giving examples, the mechanisms that regulate differentiation during development and in mature tissues.

Questions for Chapter 17

Question 17.1

Asymmetric division is an important general mechanism that underlies differentiation. Explain what is meant by this term, and explain its importance in early *C. elegans* development and in the maintenance of the intestinal epithelium.

Question 17.2

Explain how contact-mediated cell–cell interactions regulate differentiation of neurons in the neur ectoderm.

Question 17.3

Cell movements play an essential role in the early development of *Xenopus laevis*. Describe briefly the cellular movements that take place during gastrulation, and their result.

Question 17.4

How do repulsive signals determine the pathway taken by migrating neural crest cells and the trajectory of commissural axons in the developing spinal cord?

References

- Borday, C., Abadie, V., Chatonnet, F., Thoby-Brisson, M., Champagnat, J. and Fortin, G. (2003) Developmental molecular switches regulating breathing patterns in CNS, *Respiratory Physiology & Neurobiology*, **135**, pp. 121–132.
- Gurdon, J. B., Byrne, J. A. and Simonsson, S. (2003) Nuclear reprogramming and stem cell creation, *Proceedings of the National Academy of Sciences USA*, **100** (Suppl. 1), pp. 11819–11822.
- Potten, C. S., Booth, C. and Hargreaves, D. (2003) The small intestine as a model for evaluating adult tissue stem cell drug targets, *Cell Proliferation*, **36**, pp. 115–129.
- Wodarz, A. (2002) Establishing cell polarity in development, *Nature Cell Biology*, **4** (2), pp. E39–E44.

Further sources

- Gammill, L. S. and Bronner-Fraser, M. (2003) Neural crest specification: migrating into genomics, *Nature Reviews Neuroscience*, **4**, pp. 795–805.
- Rolls, M. M. and Doe, C. Q. (2003) From embryo to axon, *Nature* (News and Views), **421**, pp. 905–906.
- Sancho, E., Batlle, E. and Clevers, H. (2003) Live and let die in the intestinal epithelium, *Current Opinion in Cell Biology*, **15**, pp. 1–8.
- Verfaillie, C. M. (2002) Adult stem cells: assessing the case for pluripotency, *Trends in Cell Biology*, **12**, pp. 502–508.
- Wolpert, L., Beddington, R., Jessell, T., Lawrence, P., Meyerowitz, E. and Smith, J. (2002) *Principles of Development* (2nd edn), Oxford University Press.

18 CELL AGEING AND SENESCENCE

18.1 Introduction

Having seen how cells divide, differentiate and move, we now turn to changes that occur during the later part of the lifespan of the organism, again focusing on animals. Everyone is familiar with some of the results of organismal (i.e. whole-organism) ageing, but how do these changes relate to cellular ageing? Do all organisms (and cells) show signs of ageing, and how does cellular ageing relate to lifespan? There are many theories of ageing, which provide explanations for the ageing process at different levels (evolutionary, organismal, cellular and molecular). In this chapter, we do not have space to discuss all these theories fully. Rather, we focus on age-associated changes at the cellular and molecular levels.

Different types of cell have different lifespans. Recall that in vertebrates, some cells, such as neurons, may survive for the entire life of the organism; others, such as some epithelial cells, have a very short lifespan and are constantly being replaced. The properties of both types of cell may change as the animal ages. Here we describe some of the changes that occur in cells during ageing. We consider how these changes occur and also, briefly, the implications of these changes for tissue function.

In this chapter, we shall build on material that was covered in S204, Book 3 *The Core of Life*, Vol. II, Section 10.5, which provides background for the topics covered here.

A number of model organisms have been used to study ageing. The lifespans of these organisms vary; for example, the mean lifespan of *C. elegans* is 15 days, while that of *Drosophila* is 40 days. The mean lifespan of mice varies according to the strain, but is about 27 months.

Other properties of these organisms influence the study of ageing. For example, no cell division takes place in adult *C. elegans* and *D. melanogaster*; all their cells (apart from the germ cells) are *post-mitotic*. Vertebrate tissues, however, are composed of both post-mitotic cells, such as neurons and skeletal muscle cells, and cells that continue to divide in adults, such as epithelial cells (in addition to germ cells).

- ☐ The short lifespans of some of these model organisms have clear advantages for the study of ageing; however, there are also some disadvantages in using them. Can you suggest any?
- ☒ Adult *C. elegans* and *D. melanogaster* lack the range of differentiated tissues found in vertebrates. It could also be argued that, even for mammalian model species (such as mice), those that have a short lifespan are not a good model for long-lived species, such as humans.

Early geneticists set up large breeding programmes at the beginning of the 20th century to generate inbred strains as a source of genetically reproducible experimental animals such as mice and rats. Inbreeding made the different lines homozygous for different alleles of genes that differed in the outbreeding founding population. Usually, inbred lines are made and propagated by brother–sister matings. After 20 generations (5 years) of brother–sister matings, an inbred line should be >98.6% homozygous for any genes that were segregating in the founder population. The genetic homogeneity of these inbred strains reduces variability between experiments.

A very important issue that it is essential to consider in the study of ageing is the *variability* of the effects of ageing – both between individuals of the same species (even individuals of inbred laboratory strains), and between individual cells within a single tissue type.

As for other areas of cell and molecular biology, the use of genetic techniques has provided much information about the mechanisms of cellular ageing. While many such studies cannot be performed on humans, there are a number of human conditions in which some aspects of ageing occur prematurely. These rare conditions are known as **progerias** or **progeroid syndromes** (from the Greek meaning ‘early ageing’); examples are Werner’s syndrome and ataxia telangiectasia (Section 9.8.8). The study of cells from individuals with these conditions has provided important information about ageing, as outlined in Section 18.5.

The occurrence of progeroid syndromes shows that mutations in certain genes can result in *premature* ageing. Note that, as yet, no genes that when mutated *delay* ageing or prolong longevity in humans have been identified. This fact is a powerful argument against the once-popular notion that ageing is in some way programmed and that ‘ageing genes’ exist. Rather, biologists believe that organisms are programmed to survive, and particularly to optimize their chances of successful reproduction. Any genes that result in the postponement of ageing would, according to current evolutionary theory, exert this effect only as a ‘by-product’ of their beneficial effects on reproductive ‘fitness’ earlier in life. This point is the basis for currently accepted theories of ageing.

It is now generally believed that ageing is the result of a combination of processes, acting on all cellular components; this is the ‘**network theory**’ (Kowald and Kirkwood, 1996). It is also widely accepted that free radicals (Section 18.2) are a major cause of molecular and cellular damage, which affects many cellular components and processes during ageing; this is the ‘**free radical theory**’ of ageing (Harman, 1956). Genes that confer increased resistance to free radicals and other forms of cellular stress protect against molecular and cellular damage, and may therefore also have the effect of delaying cellular ageing. If the products of these genes also exert a beneficial effect during the period of reproduction, they will be selected for and passed on to subsequent generations.

What are the cellular processes that are affected in ageing cells? Perhaps not surprisingly, given the complexity of cells, many changes, affecting most types of molecule and cellular component, have been detected in ageing cells. Examples include:

- ▶ differences in the expression of proteins and mRNA;
- ▶ changes in both the nuclear and mitochondrial genomes;
- ▶ changes in energy metabolism;
- ▶ modifications in protein processing;
- ▶ accumulation of aggregated or damaged proteins and lipids;
- ▶ cessation of division of proliferative cells;
- ▶ functional alterations, both general and cell type-specific.

In this chapter, we briefly describe some of the main features of cellular ageing.

Summary of Section 18.1

- 1 Most types of molecule and cellular component of animal cells are affected during ageing, and different types of animal cells have different lifespans.
- 2 Cellular ageing is studied using cells from model organisms that have very different lifespans.
- 3 Two of the many theories of ageing are the network theory, which states that cellular ageing is the result of a combination of processes, and the free radical theory, according to which free radical damage is the cause of molecular and cellular ageing.
- 4 Genes that confer protection against free radicals and other stresses may have a positive effect against molecular and cellular damage occurring during ageing.

18.2 Free radicals and ageing

18.2.1 What are free radicals and how are they generated?

Free radicals are atoms or molecules that possess unpaired electrons. Free radicals are energetically unstable; many types of free radical are hence very reactive and combine with any nearby molecules, causing oxidative damage (explained in Section 18.2.2) to nucleic acids, proteins and lipids.

There are many types of free radical. Perhaps of most interest to biologists are oxygen-based radicals, which together with non-radical oxidants such as hydrogen peroxide (H_2O_2) and lipid peroxide (LOOH) are often called **reactive oxygen species (ROS)** (introduced in Book 1, p. 216). Some types of ROS are generated as by-products of oxidative metabolism, but others are generated by other cellular reactions. For example, the peroxynitrite anion (ONOO^-) is produced by the breakdown of the signalling molecule nitric oxide (NO), itself a free radical (so may also be represented as $\text{NO}^\bullet$; see S204, Book 3, Vol. II, Chapter 6). Nitric oxide and peroxynitrite can be considered not only as ROS but also as reactive nitrogen species (RNS); that is, free radicals and non-radical oxidants that are derived from nitrogen-based molecules. Some metal ions (see Section 2.4.6), such as iron and copper, also possess unpaired electrons (which is why these ions form part of protein complexes that play essential roles in electron transfer in cells). These metal ions are thus, by definition, free radicals and in some circumstances can contribute to damaging reactions in cells. Other non-oxygen-based free radicals such as carbon-centred radicals and thiol (sulfur-based) radicals also occur in cells, but we shall not consider them in detail here. Some of the most widely occurring free radicals and reactive non-radicals are shown in Table 18.1.

As mentioned above, the main source of ROS in eukaryote cells is oxidative metabolism. In animals, about 85–90% of the oxygen that is taken up is used in this process. You should recall from Book 1, Chapter 4, that all aerobic organisms generate ATP by utilizing electron transport along a chain of carrier proteins (the electron transport chain, ETC), to the final electron acceptor, molecular oxygen. The process is called oxidative phosphorylation (Section 4.5.1).

- ☐ What is the site of oxidative phosphorylation in animal cells?
- ☒ The inner membrane of the mitochondrion (Book 1, Chapter 4, Figure 4.9a).

Table 18.1 Some examples of free radicals and reactive non-radicals that commonly occur in cells.

Free radicals		Reactive non-radicals	
superoxide	$\text{O}_2^{\bullet-}$		
hydroxyl	$\text{OH}^{\bullet}$	hydrogen peroxide	H_2O_2
peroxyl	$\text{ROO}^{\bullet}$	lipid peroxide	LOOH
alkoxyl	$\text{RO}^{\bullet}$	peroxynitrite	ONOO^-
hydroperoxyl	$\text{HOO}^{\bullet}$		
lipid peroxyl	$\text{LOO}^{\bullet}$		
nitric oxide	$\text{NO}^{\bullet}$		
carbon-centred radicals*	$\text{R}_3\text{C}^{\bullet}$		
thiol radicals	$\text{RS}^{\bullet}$		

*Carbon-centred radicals tend to react with oxygen to form peroxyl radicals (see Section 18.3.2).

During oxidative metabolism, each oxygen molecule accepts four electrons and combines with four protons to form two molecules of water:



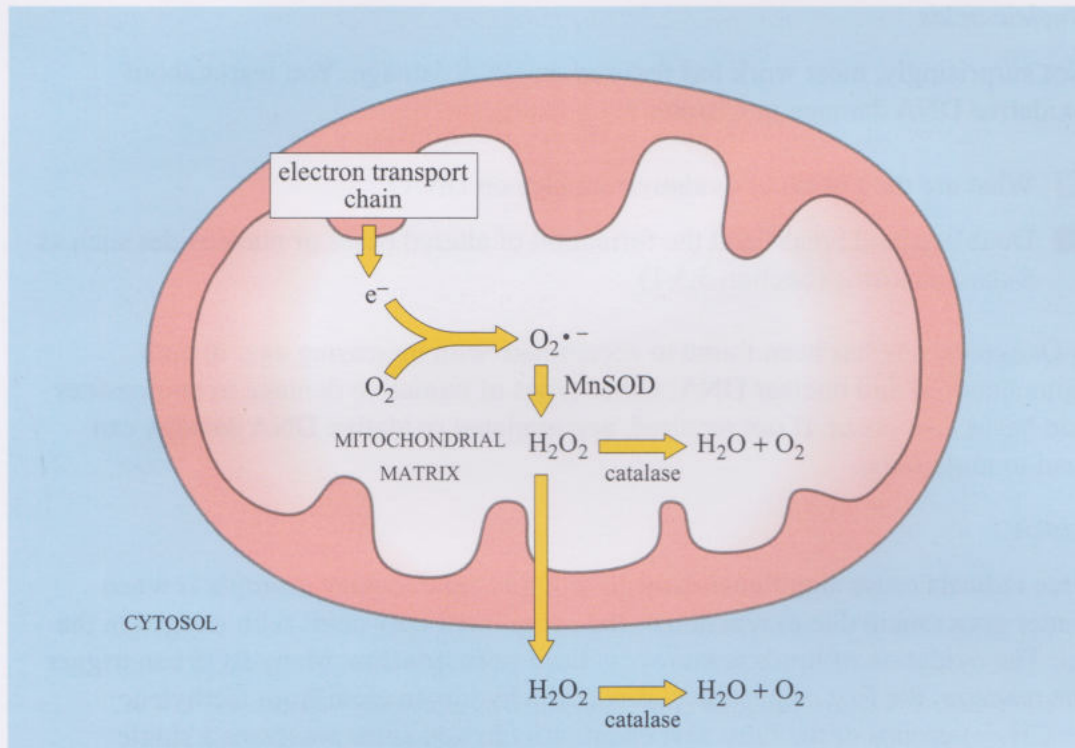
Note that, because there are *two* unpaired electrons in the oxygen molecule, it is more correctly termed a *biradical*. (A fuller explanation can be found in S205 *The Molecular World*, Book 6, pp. 54–6.)

Molecular oxygen (O_2) itself has two unpaired electrons and behaves like a free radical. The two unpaired electrons of molecular oxygen (O_2) mean that it is very reactive (which is why, for example, substances rust and burn when exposed to oxygen in the air). Molecular oxygen is a strong oxidant; however, it cannot accept four electrons simultaneously. So, in the mitochondrion, its reduction to water occurs in a stepwise manner, in reactions catalysed by the final protein complex in the ETC, the cytochrome oxidase complex (also called complex IV; Book 1, Figure 4.9a).

Cytochrome oxidase is very efficient; partially reduced oxygen species are held within the complex by haem iron and copper ions that are bound to the protein complex. The electron carriers earlier in the ETC, however, are less efficient, and some electrons leak across the mitochondrial membrane, and are able to combine *singly* with molecular oxygen, to form **superoxide radicals** ($\text{O}_2^{\bullet-}$), as shown in Figure 18.1.

The identity of the components of the ETC that allow the ‘leak’ of electrons remains controversial; there is evidence that complex I components, some cytochromes and ubiquinone may be involved. It has been estimated that of the oxygen that is reduced in the mitochondria, approximately 1–3% is only partially reduced, picking up a single leaked electron, to form superoxide radicals.

Superoxide is, in fact, a weaker radical than oxygen; however, it is rapidly converted into **hydrogen peroxide** (H_2O_2) by the action of the mitochondrial form of the enzyme **superoxide dismutase**, which is known as MnSOD because it has manganese (Mn) as its cofactor (Figure 18.1 and Table 18.2).

**Figure 18.1**

The formation of ROS in mitochondria. Electrons that leak from the ETC across the inner mitochondrial membrane reduce molecular oxygen (O_2), resulting in the formation of superoxide radicals ($O_2^{\bullet -}$). These superoxide radicals are converted to hydrogen peroxide (H_2O_2) by the mitochondrial form of the enzyme superoxide dismutase (known as MnSOD; see Table 18.2). Hydrogen peroxide can cross membranes; it is inactivated by the enzyme catalase (see Table 18.2), which is present in both the mitochondrial matrix and the cytosol. (Note that, for simplicity, the stoichiometry of the reactions is not shown here; but see Table 18.2.)

☐ From Table 18.1, is hydrogen peroxide a free radical?

☒ No, it has no unpaired electrons.

Although hydrogen peroxide is not a free radical, it is considered a ROS because it undergoes a number of reactions that generate extremely reactive radicals, notably the **hydroxyl radical ($OH^{\bullet}$)** (Table 18.1). Moreover, hydrogen peroxide readily crosses membranes, and so can diffuse throughout the cell. Hydrogen peroxide is broken down by the action of another enzyme, **catalase**, which is present in both the mitochondrial matrix and the cytosol (Figure 18.1 and Table 18.2).

Although the mitochondria are regarded as the main source of ROS in aerobic cells, other important cellular sources of ROS exist. Some 10–15% of the oxygen that is taken up by a cell is *not* used in respiration, but is used in reactions catalysed by oxidase and oxygenase enzymes. Members of the cytochrome P450 superfamily are important examples of enzymes that utilize oxygen; they oxidize many substrates in the cell and also exogenous substances such as drugs and chemicals, and so play an essential role in detoxification. Some small endogenous molecules, such as the hormone adrenalin and the neurotransmitter dopamine, also react with oxygen, albeit slowly, in auto-oxidation reactions (spontaneous non-enzymatic combination with O_2). All these reactions involving oxygen can generate ROS. Finally, exogenous agents such as ionizing and ultraviolet radiation, and some chemicals, also produce free radicals.

18.2.2 What do free radicals do to molecules and cells?

The reactive nature of free radicals means that they have the potential to react with any molecule that they encounter. These reactions can alter the structure of the molecule, with the result that its function may be impaired. The molecular changes caused by ROS are hence often known as *oxidative damage*. Here we briefly review the nature of the damage caused by free radicals to nucleic acids, proteins and lipids.

Nucleic acids

Not surprisingly, most work has focused on DNA damage. You learnt about oxidative DNA damage in Chapter 5.

- ☐ What are the effects of oxidative damage on DNA?
- ☒ Double-strand breaks and the formation of altered bases or nucleosides such as 8-oxoguanosine (Section 5.5.1).

8-Oxoguanosine has been found to accumulate with increasing age, in both mitochondrial and nuclear DNA. Other types of oxidative damage to nucleosides and bases also occur. If not repaired, accumulated oxidative DNA damage can lead to mutations.

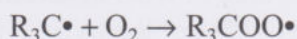
Lipids

Free radicals cause significant damage to lipids; an everyday example is when butter goes rancid due to reaction of the component fatty acids with oxygen in the air. The oxidation of lipids is known as **lipid peroxidation**. Many ROS can trigger the reaction; the first step is the removal of a hydrogen atom from methylene ($-\text{CH}_2-$) groups of the fatty acyl chain. A hydrogen atom possesses a single electron, so the remaining carbon atom has an unpaired electron. As an example, we show the reaction between a methylene group-containing molecule and a hydroxyl radical:

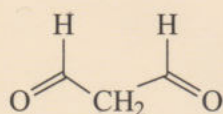


- ☐ What type of radical is a carbon chain containing the $-\text{C}\cdot\text{H}-$ group?
- ☒ A carbon-centred radical (see Table 18.1).

Carbon-centred radicals usually combine with oxygen, to form peroxy (or peroxy) radicals:



Peroxy radicals can then remove hydrogen atoms from other lipid molecules, such as adjacent fatty acyl side-chains, thus creating more peroxy radicals, and setting up a *chain reaction*. A variety of products may be formed; an example is malondialdehyde (see margin), which can go on to have damaging effects on other components of the cell.



malondialdehyde

Proteins

Free radicals can damage proteins in a number of ways, both directly and indirectly by the action of the products of lipid oxidation. The direct effects of free radicals on amino acid residues in proteins depend upon the radical and the amino acid. We cannot detail all these reactions here, but will briefly describe a few common effects of free radicals on amino acids.

One common effect of some types of ROS on amino acids is the formation of **carbonyl groups** ($>C=O$) within proteins. Another common effect is that of RNS on tyrosine, forming **3-nitrotyrosine**. Some other amino acids are also altered by reaction with RNS. Thiol groups, found in cysteine and methionine, readily react with both ROS and RNS. When proteins are oxidized by free radicals, **amino acid radicals** (e.g. Met $\bullet$) can be formed, which can go on to oxidize other amino acid residues. These are just a few examples; the important point is that the effect on the protein will depend upon how many and which residues are affected.

- ☐ Oxidation of which amino acids will affect enzyme function?
- ☒ Those that affect activity, i.e. those within the activation loop or those that modulate activity by facilitating binding to substrates or cofactors. Some oxidation of other amino acids can be tolerated without affecting function. The functional effect will depend upon the enzyme concerned, and the extent of the damage.

How do cells, especially long-lived cells such as neurons, survive this onslaught of oxidative damage? The answer is that there is, in normal circumstances, a balance between free radical generation and the action of cellular antioxidant defences. These defences are not completely efficient, however, so some damage does occur with time. Additionally, a particular situation may provoke a shift in this balance, so that increased levels of free radicals are present; this situation is often termed **oxidative stress**. Oxidative stress occurs in several circumstances; examples include inflammatory diseases, severe depletion of dietary antioxidants, mutations in antioxidant enzymes and exposure to radical-generating exogenous agents. Smoking is a good example of the latter.

18.2.3 Cellular defences against free radical damage

The evolution of aerobic organisms was accompanied by the evolution of antioxidant defences, which enabled organisms to survive the damaging effects of oxygen or its derivatives. Antioxidant defences are often classified as either *primary* or *secondary*. Primary defences limit the availability of free radicals, by removing or inactivating them; secondary defences repair molecules damaged by free radicals.

Primary antioxidant defences

Primary antioxidant defences are of three main types: (1) small-molecule antioxidants that 'scavenge' ROS and RNS; (2) proteins that bind metal ions, thus limiting their availability; (3) antioxidant enzymes, which convert free radicals to less reactive or inactive forms. Examples of the primary antioxidant defences are shown in Table 18.2.

Table 18.2 Primary antioxidant defences.

Antioxidant	Type and location of molecule	Simplified outline of function
vitamin E (α -tocopherol)	Lipid-soluble organic molecule. Localized in membranes, abundant in the thylakoid membranes of chloroplasts.	Several types of antioxidant activity. Important in limiting damage to membranes during lipid peroxidation. The α -tocopherol radical that results is reduced to its original form by another antioxidant, vitamin C.
vitamin C (ascorbic acid)	Water-soluble organic molecule. Localized in the cytosol.	Many roles, including antioxidant role in deactivating many ROS. Also reduces oxidized α -tocopherol.
glutathione (GSH)	Tripeptide (Glu–Cys–Gly) when reduced, but on oxidation, dimerizes by formation of a disulfide (S–S) bond (Chapter 3) between the two cysteine residues. Present in the cytosol and cell organelles.	Reacts with a number of oxidizing agents, including oxygen and organic carbon-centred free radicals, e.g. $R_3C\cdot + G-SH \rightarrow R_3CH + GS\cdot$ then: $G-S\cdot + G-S\cdot \rightarrow G-S-S-G$ Also acts as an essential reductant coenzyme, providing electrons to a variety of enzymes.
superoxide dismutase (SOD)	Enzyme Different forms are present in the mitochondrial matrix (MnSOD, cofactor manganese) and the cytosol (CuZnSOD, cofactors copper and zinc).	Catalyses the formation of hydrogen peroxide from superoxide radicals: $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$
catalase	Enzyme Present in the mitochondrial matrix and the cytosol.	Catalyses the breakdown of hydrogen peroxide to water and oxygen: $2H_2O_2 \rightarrow 2H_2O + O_2$
glutathione peroxidase	Enzyme Present in the mitochondrial matrix and the cytosol.	Catalyses the breakdown of hydrogen peroxide, by reaction with glutathione: $H_2O_2 + 2G-SH \rightarrow 2H_2O + G-S-S-G$

Secondary antioxidant defences

Secondary defences are repair enzymes, such as those that repair DNA damage (Chapter 9), the protein degradation systems (Chapter 11) and stress response proteins (Chapter 11). Expression of some of these proteins is upregulated following oxidative stress. For example, there is evidence that, in some instances, the expression of heat shock proteins (Hsps; see Chapter 3, Section 3.3.1) is induced upon oxidative damage, to correct protein misfolding, and may prevent further oxidative damage.

Box 18.1

Studying free radicals and the damage that they produce

Generation of free radicals

Because of their highly reactive nature, free radicals are usually studied indirectly. Generation of free radicals can be detected by loading of cells with, for example, dihydrorhodamine, which is itself non-fluorescent, but is converted to the fluorescent compound rhodamine upon exposure to free radicals (Figure 18.2). Other methods quantify free-radical reaction products in cells, e.g. the lipid peroxidation product malondialdehyde.

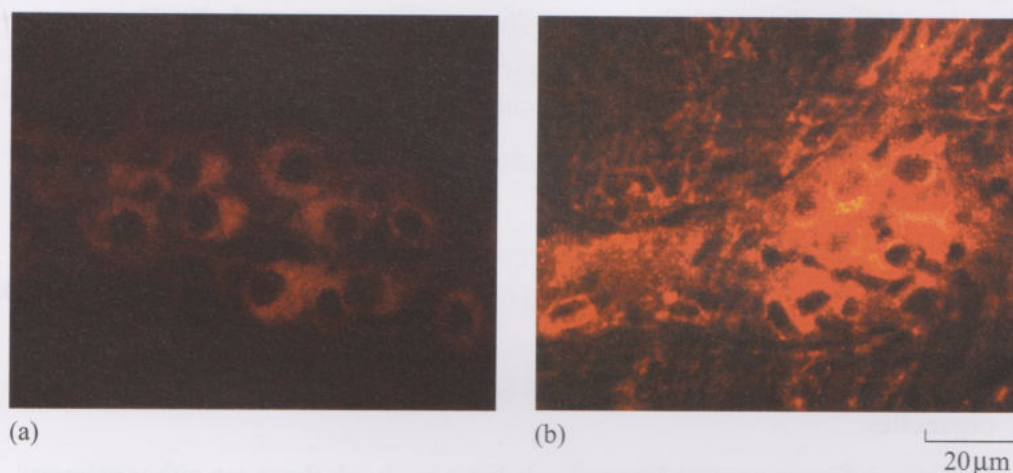


Figure 18.2 Rhodamine fluorescence induced by the action of endogenous free radicals in tissue samples incubated with dihydrorhodamine. (a) Young (6-month) and (b) old (24-month) rat enteric ganglia (groups of neurons in the gut). The tissues were loaded with dihydrorhodamine and examined microscopically two hours later. The fluorescence in the neurons in the old tissue is much brighter than that in the young tissue, indicating an increase in free radical generation. (Courtesy of Tim Cowen)

Effects of free radicals

The changes to macromolecular structure that are produced by free radicals are studied by a range of chemical and biochemical techniques. The effects of free radicals are studied by altering the balance of free radicals within cells or whole organisms. This is usually achieved either by manipulating the expression of antioxidant defences by genetic techniques, or by exposing cultured cells or model organisms to chemicals that induce oxidative stress (e.g. hydrogen peroxide, paraquat) or to free radical scavengers (e.g. α -tocopherol, Table 18.2), and determining their specific (e.g. protein folding) or generalized (e.g. cell toxicity) effects.

Paraquat is a widely used insecticide. Within cells, it is first oxidized and then reduced as a result of the activity of two enzymes with opposing actions. The result is a cycle of reactions referred to as a 'futile redox cycle', in which ROS are generated as by-products.

18.2.4 What is the evidence for a causal role of free radicals in cellular ageing?

Finally, we turn to the crucial question: what is the evidence for free radicals being the (or one) causal factor in cellular ageing? There have been numerous reports of increased levels of oxidatively damaged macromolecules in samples taken from ageing laboratory animals and from humans. This evidence alone, however, does not *prove* that oxidative damage is responsible for the changes in tissue function that occur with age. More recent evidence, however, in which the effects of agents or manipulations that change the balance of free radicals within cells or organisms have been studied, supports the hypothesis that free radicals play an important part in ageing. For example, SOD/catalase mimetics protect *C. elegans* against paraquat-induced oxidative stress and also extend lifespan (Sampayo *et al.*, 2003).

A mimetic is a synthetic agent that mimics the action of a natural agent, such as an enzyme.

Summary of Section 18.2

- 1 Free radicals are atoms or molecules that possess unpaired electrons and are therefore very reactive.
- 2 There are many naturally occurring free radicals; one major source within cells is oxidative phosphorylation, which takes place in the inner mitochondrial membrane.
- 3 Free radicals cause oxidative damage to all types of molecules and cell organelles.
- 4 Cellular defences against free radicals have evolved; these are either primary or secondary defences. Primary defences include small-molecule radical scavengers, proteins that bind metal ions, and antioxidant enzymes. Secondary defences include repair enzymes, stress response proteins and protein degradation systems.

18.3 Ageing of mitotically active cells: replicative senescence

Replicative senescence was introduced in S204, Book 3, Vol. II, Section 10.5.4.

18.3.1 Cells have a limited replicative lifespan

You may recall from previous study that normal, non-transformed (i.e. non-tumorigenic) cells have a limited replicative lifespan in culture; that is, they cannot continue to divide indefinitely, but after a number of cell divisions, they withdraw from the cell cycle and enter a state known as **replicative senescence**, which is irreversible.

- ☐ Senescent cells do not divide. Name two other situations in which cells do not divide.
- ☒ In quiescence (the G0 phase), cells temporarily leave the cell cycle (Chapter 8). Some terminally differentiated cells also do not divide (Chapters 8 and 17).

Before describing what is currently known about replicative senescence, its causes and effects, we consider how it is studied.

Box 18.2 How is replicative senescence studied?

Study of replicative senescence *in vitro*

Replicative senescence has predominantly been studied using cultured cells, because of the obvious difficulties of studying cell proliferation over long periods *in vivo*. Cultures of human fibroblasts, obtained by biopsy, have most often been used, although other types of human cells, and animal cells, particularly from rodents, have also been examined.

The proliferative capacity of cells in culture is assessed by measuring the number of times the total population of cells in the culture doubles; a parameter known as **population doubling (PD)**. The number of PDs that cells of a particular type can undergo *in vitro* before all the cells in the culture become senescent is known as the **Hayflick limit**, after Leonard Hayflick, who, with Paul Moorhead, first reported the phenomenon in 1961.

How are PD numbers determined? Usually, they are estimated by counting the number of cells in culture. A known number of cells are placed in a culture dish and allowed to divide until they completely cover the surface of the dish, a situation known as *confluence*. At confluence, the cells are *passaged*; that is, they are removed from the dish, counted and re-plated, at their original density, onto several new dishes. This process is repeated as necessary until the cells stop dividing. Depending upon the cell type and the culture conditions, cultures may be passaged once or twice each week, or less frequently. The increase in the number of cells at each passage can be calculated and expressed as the number of times the original population doubled in size. Repeating this calculation at each passage and adding the values obtained gives a cumulative value. The cumulated value when the cells reach senescence is the final number of PDs for that cell culture.

- ☐ Can you think of any flaws inherent in this technique?
- ☒ There are several flaws. (1) The method cannot take into account any cells that may die, either in culture, or during the passaging process. (2) Some cells in the culture may divide more

frequently than others, and some may not divide at all; these situations would not be detected simply by counting. In other words, as its name suggests, the method can only be used to assess changes in the *population* of cells but gives no information about replication of individual cells. (3) The behaviour of cells in culture may be very different from that of cells in intact tissues *in vivo*.

- ☐ Suggest some additional methods that would allow you to estimate (a) the proportion of cells that are proliferating in a culture dish, and (b) whether or not the cell population is in any other way heterogeneous.
- ☒ (a) You could introduce a label that is incorporated into newly synthesized DNA, such as ³H-thymidine or BrdU (Chapter 9, Box 9.1, and Section 17.4.1). (b) You could examine the cells by phase contrast microscopy or examine the expression of proteins expressed only by cells that are dividing, such as DNA replication enzymes.

Studies of the replicative potential of cells in culture hence often use a combination of techniques to measure not only PD, but also the *proportion* of cells replicating within a population at different stages of culture, and the *rate* at which cells become senescent. Figure 18.3 shows two cultures of the same cell type but at different PD values.

The proportion of proliferating cells in culture has been measured using an antibody that recognizes an antigen expressed only by proliferating cells. The fraction of proliferating cells falls steadily from about 60% to around 2–5% over 120 days in culture; this represents an approximate loss of 1% of the proliferating fraction for every population doubling.

Changes occurring in senescent cells can impact on the histochemical reaction for the enzyme β -galactosidase, so that, under specific reaction conditions, β -galactosidase activity can only be detected in senescent cells. This so-called senescence-associated β -galactosidase (SA- β gal) reaction has been used to identify senescent cells (Figure 18.4).

Figure 18.3

Phase contrast images of (a) non-senescent (at 10–15% of their maximal PD) and (b) senescent (more than 95% of maximal PD) human dermal (skin) fibroblasts. These cells typically undergo about 65 cumulative population doublings over 120 days *in vitro*. Note that senescent cells are larger than non-senescent cells, and their nuclei are bigger too. (Courtesy of Dr I. Kill)

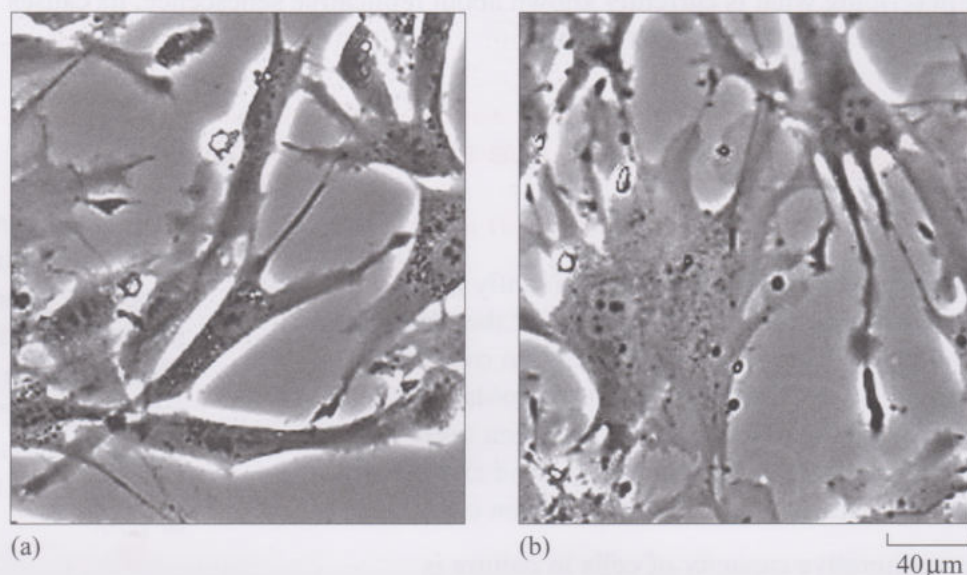


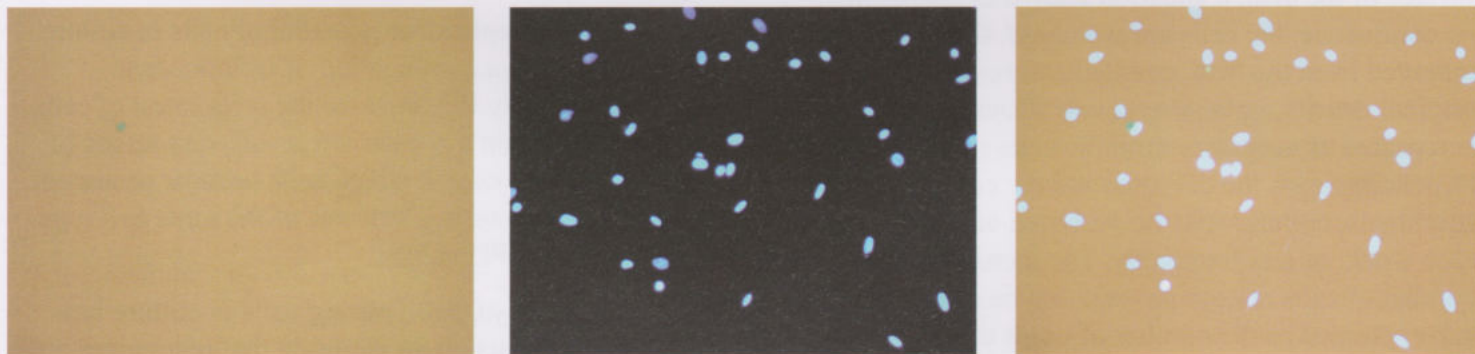
Figure 18.4 (a) SA- β gal staining of non-senescent and senescent human dermal (skin) fibroblasts in culture. (b) The cultures were counterstained with DAPI (diamidino-2-phenylindole, a reagent that binds to double-stranded DNA, forming a fluorescent complex), to show the nuclei of all cells present. Superimposed images (a) and (b) are shown in (c). Note that some cells in the 'senescent' cultures are SA- β gal-negative (one of them is identified by an arrow.) (Courtesy of Dr I. Kill)

Despite the inherent difficulties with the technique of cell culture, much useful information about cell replication and its control has been obtained from studies of cultured cells.

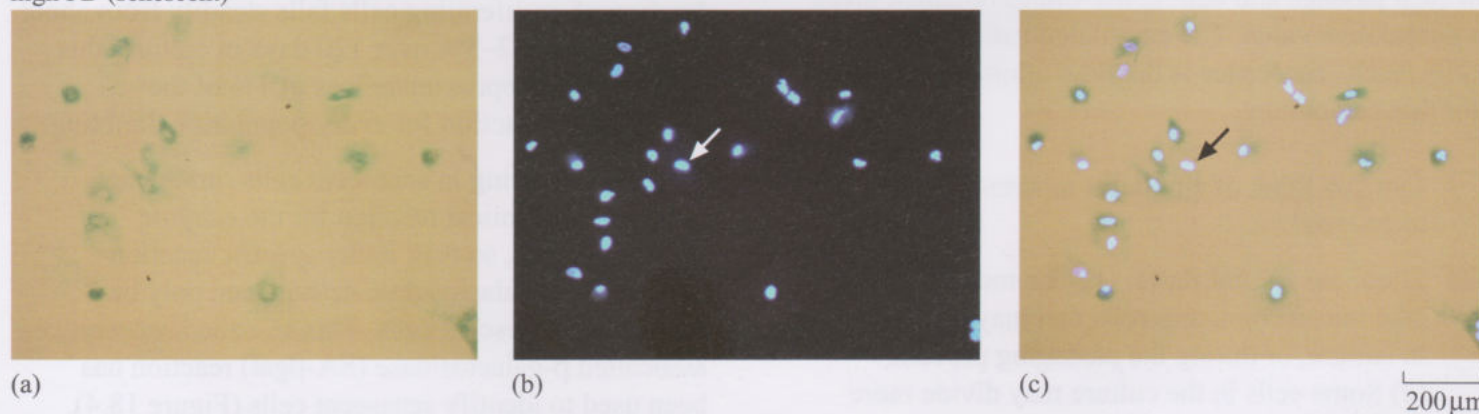
Study of replicative senescence *in vivo*

Replicative senescence is difficult to study *in vivo*. Although cell proliferation can be studied by pulsing with ^3H -thymidine or BrdU in intact tissues (such as the gut epithelium; see Section 17.4.1), the approach is difficult in tissues in which there is only a slow turnover of cells.

low PD



high PD (senescent)



The early observation that cells isolated from tissues from the oldest individuals divide fewer times than cells from very young individuals aroused great excitement amongst researchers. It was suggested that cells have a limited replicative lifespan, and must therefore possess some kind of intrinsic 'replication counter' (sometimes called a 'replicometer') that is able to measure the number of divisions that the cell has undergone and then, after a set number of divisions, trigger senescence. The phenomenon also led to the idea that replicative senescence could underlie the changes that occur in many tissues during ageing.

Other evidence supported the notion that the replicative lifespan of proliferative cells was in some way related to organismal ageing. First, cells from long-lived animals such as humans and tortoises tended to exhibit a greater number of PDs *in vitro* than cells from short-lived animals such as mice. Second, cells from individuals with progeroid syndromes, such as Werner's syndrome, underwent fewer PDs than those from other individuals.

This attractive and simple idea, however, presents a number of problems. We have already considered some of the flaws inherent in attempting to draw conclusions from the study of populations of cells in culture (Box 18.2). The cultured cells are heterogeneous with respect to proliferative potential; detailed analysis shows that some cells are senescent at early stages of culture, even in cultures obtained from young individuals. Close examination of the data also reveals that there is not actually much difference in the replicative lifespans of cultured human cells between young and elderly individuals; the main differences are between cells derived from fetuses and those from people over 80 years of age. Senescence can also be triggered by some culture conditions, a phenomenon that we consider in Section 18.3.3.

Another important question is whether or not cells undergo replicative senescence *in vivo*. Fibroblasts, for example, do not turn over rapidly *in vivo*. Typically, however, human dermal (skin) fibroblasts have a lifespan *in vitro* of around 50 PDs, more than is needed for continued replication throughout life. In other words, there is a *proliferative reserve capacity*. This notion is supported by the observation that only some senescent cells are seen in skin sections, and that some fibroblasts obtained from aged individuals have similar PDs to those from young individuals. In situations of injury or stress, however, it may be that the reserve capacity is only just, or not quite, enough to restore normal tissue structure and function.

Although the turnover rate of many other cell types, such as endothelial cells that line blood vessels, is slow *in vivo*, some other cell types do turn over more rapidly, and must retain this proliferative capacity throughout the life of the organism.

- ☐ What cells must retain proliferative capacity throughout the organism's life?
- ☒ Stem cells and their progeny cells (Chapter 17).

Studies of intestinal stem cells in the crypts of mouse intestine (Section 17.4.1) have shown that crypt stem cells from older animals respond differently to injury to those from younger animals. Stem cells from older animals showed an increased apoptotic response following exposure to low doses of ionizing radiation. The regenerative capacity of these stem cells after high doses of radiation was also lower in the older mice. Much remains to be learnt about replicative senescence of stem cells; however, this example shows that changes in the properties of stem cells may impact on tissue ageing.

18.3.2 Changes in gene expression by senescent cells

What are the main features of senescent cells? Do the properties of cells change upon senescence, so that they are able to contribute to tissue ageing? Certainly senescent cells in culture remain metabolically active, but inspection of Figure 18.3 shows that their morphology also changes.

- ☐ How does the appearance of senescent cells change in culture over increasing PDs?
- ☒ They are larger, and their nuclei are bigger (Figure 18.3).

As well as an increase in cell and nuclear size in culture, senescent cells exhibit cytoskeletal alterations, increases in lysosomal size and mitochondrial changes. In addition, some types of cells are resistant to apoptosis when senescent, whereas others are more sensitive. The transcription of a number of genes also changes when cells become senescent. Expression of some genes increases, that of others decreases; some examples are shown in Table 18.3.

Table 18.3 Examples of changes in gene expression by senescent cells.

Expression increased in senescent cells	Expression decreased in senescent cells
p21CIP (Chapter 8, Section 8.3.5)	cyclins A and D (Chapter 8, Section 8.3)
collagenase (enzyme that breaks down collagen; Chapter 16)	Fos (Chapter 13, Section 13.3.7)
TIMP-2 (tissue inhibitors of metalloproteases; Chapter 16, Section 16.7)	α 1 (III)-procollagen (a precursor of collagen)
MMP3, MMP10 (matrix metalloproteases; Chapter 16, Section 16.7)	interleukin-6 (a cytokine)
fibronectin (Chapter 16, Section 16.2.4)	TGF β (Chapter 17, Section 17.2.4)
interleukin-1 β (inflammatory cytokine)	IGF-1 (insulin-like growth factor 1; Section 18.6)
ICAM-1 (Chapter 16, Section 16.2.3)	
IGF-binding proteins (proteins that bind to and so modulate the effects of IGFs)	

- ☐ What is the implication of the reduced expression of cyclins A and D by senescent cells?
- ☒ Reduced levels of cyclins A and D will inhibit progression of the cell cycle (see Figure 8.22b).
- ☐ How does a reduction in cyclin D levels result in inhibition of the cell cycle?
- ☒ Reduction in cyclin D levels will reduce levels of the cyclin D–Cdk complex, and hence its activity. Cyclin D–Cdk normally phosphorylates and thereby inactivates the Rb protein, inducing cell proliferation. Loss of cyclin D–Cdk activity therefore leads to a build-up of active Rb, which inhibits cell proliferation (Chapter 8, Figure 8.25).

Some of the changes in gene expression seen upon the onset of senescence are related to the withdrawal of the cell from the cell cycle; examples are the upregulation in expression of the growth inhibitor p21CIP (Chapter 8, Section 8.3.5), and the downregulation of cyclins A and D expression. Other changes in gene expression are unrelated to replication. An important point is that gene expression changes induced by senescence are *not* identical in different cell types, although some general patterns emerge. For example, in senescent fibroblasts, there is an increase in expression of some of the genes that are upregulated in inflammation, such as those encoding proteases and cytokines, indicating that, if such genes are expressed by senescent cells *in vivo*, an inflammatory reaction could be initiated, thereby affecting nearby cells and/or even whole tissues.

18.3.3 What is the mechanism by which cells become senescent?

It is now known that there are several different events that can cause cells to become senescent (Figure 18.5). In culture, these events include shortening of telomeres (Chapter 9), some kinds of DNA damage (including oxidative stress, radiation-induced damage and double-strand breaks), decondensation of chromatin (which can be induced by pharmacological agents such as those that target histone deacetylases, or by manipulation of the expression of chromatin remodelling proteins; Chapter 10), overactivation of some mitogenic stimuli (such as those that activate the MAP kinase pathway; Chapter 13), and activation of some oncogenes (e.g. those encoding the Ras and Raf signalling molecules; Chapter 13, Section 13.3). (Oncogenes will be described in more detail in Chapter 19.)

For many years, it was widely accepted that telomere shortening was the cause of cellular senescence.

- ☐ What are telomeres?
- ☒ Telomeres are repeat sequences at the ends of chromosomes; they are associated with proteins and protect the ends of chromosomes. In vertebrates, the telomere repeat sequence is 5'-TTAGGG-3' (Chapter 9, Section 9.5).

Telomere shortening does not always occur in senescent cells; in fact, it is now known that there are both **telomere-dependent** and **telomere-independent** pathways to replicative senescence.

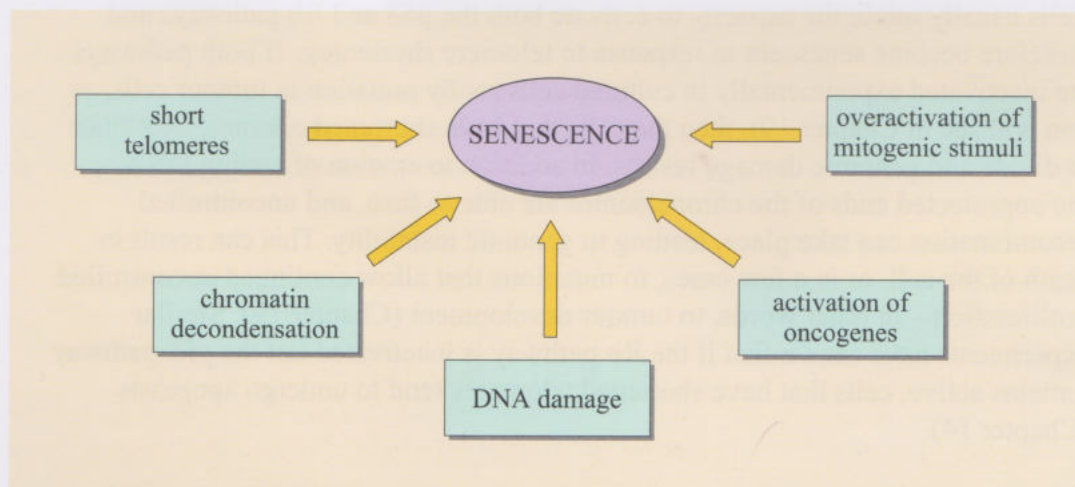


Figure 18.5
Events that can trigger replicative senescence in cultured cells.

The telomere hypothesis

When it was discovered that telomeres shorten with increasing PD, the mechanism underlying the intrinsic replication counter (Section 18.3.1) in cells seemed to have been identified.

- ☐ What happens to telomeres at each round of DNA replication in most somatic cells, and why?
- ☒ At each round of replication, the telomeres become shorter, because of the end-replication problem (Section 9.8).
- ☐ What prevents the shortening of telomeres in germ cells and some stem cells?
- ☒ Germ cells and some stem cells express the enzyme telomerase, which synthesizes new telomeric DNA (Section 9.5.2).

Telomerase is the enzyme that synthesizes new telomeric DNA. It is a reverse transcriptase, and consists of a regulatory and a catalytic subunit.

According to the telomere hypothesis of replicative senescence, somatic cells become senescent when their telomeres reach a critically short length. The onset of senescence thus prevents chromosome rearrangement and erosion of coding DNA, which would have potentially disastrous consequences for the cell (as genetic material would be lost from the ends of their chromosomes).

In order for the telomere hypothesis to be upheld, there must be a mechanism that detects the critical shortening of the telomeres, and signals this event to the molecular regulators of the cell's replication machinery. Evidence implicates several molecules that you have already encountered (ATM, p53 and downstream components of the cell cycle checkpoints) as detectors of critical telomere length. Some aspect of telomere shortening is detected by ATM in cells (we return to exactly what is detected, later in this section).

- ☐ Which proteins are affected downstream of ATM to cause cell cycle arrest?
- ☒ Chk1 and 2, which then signal to both the phosphatase Cdc25 and p53 (Chapter 8, Section 8.3.5).

The mechanism by which detection of telomere shortening by ATM results in withdrawal from the cell cycle is shown in Figure 18.6.

Cells usually retain the capacity to activate both the p53 and Rb pathways and therefore become senescent in response to telomere shortening. If both pathways are inactivated experimentally in cultured cells (or by mutation in tumour cells, as you will see in Chapter 19), then the cells that have shortened telomeres continue to divide and genomic damage results. In addition to erosion of coding DNA, the unprotected ends of the chromosomes are able to fuse, and uncontrolled recombination can take place, leading to genomic instability. This can result in death of the cell, or in a few cases, to mutations that allow continued uncontrolled proliferation – in other words, to tumour development (Chapter 19). Similar experiments have shown that if the Rb pathway is inactivated but the p53 pathway remains active, cells that have shortened telomeres tend to undergo apoptosis (Chapter 14).

Note that the two alternative terms *genomic instability* and *genetic instability* are used in this course and also in the primary literature.

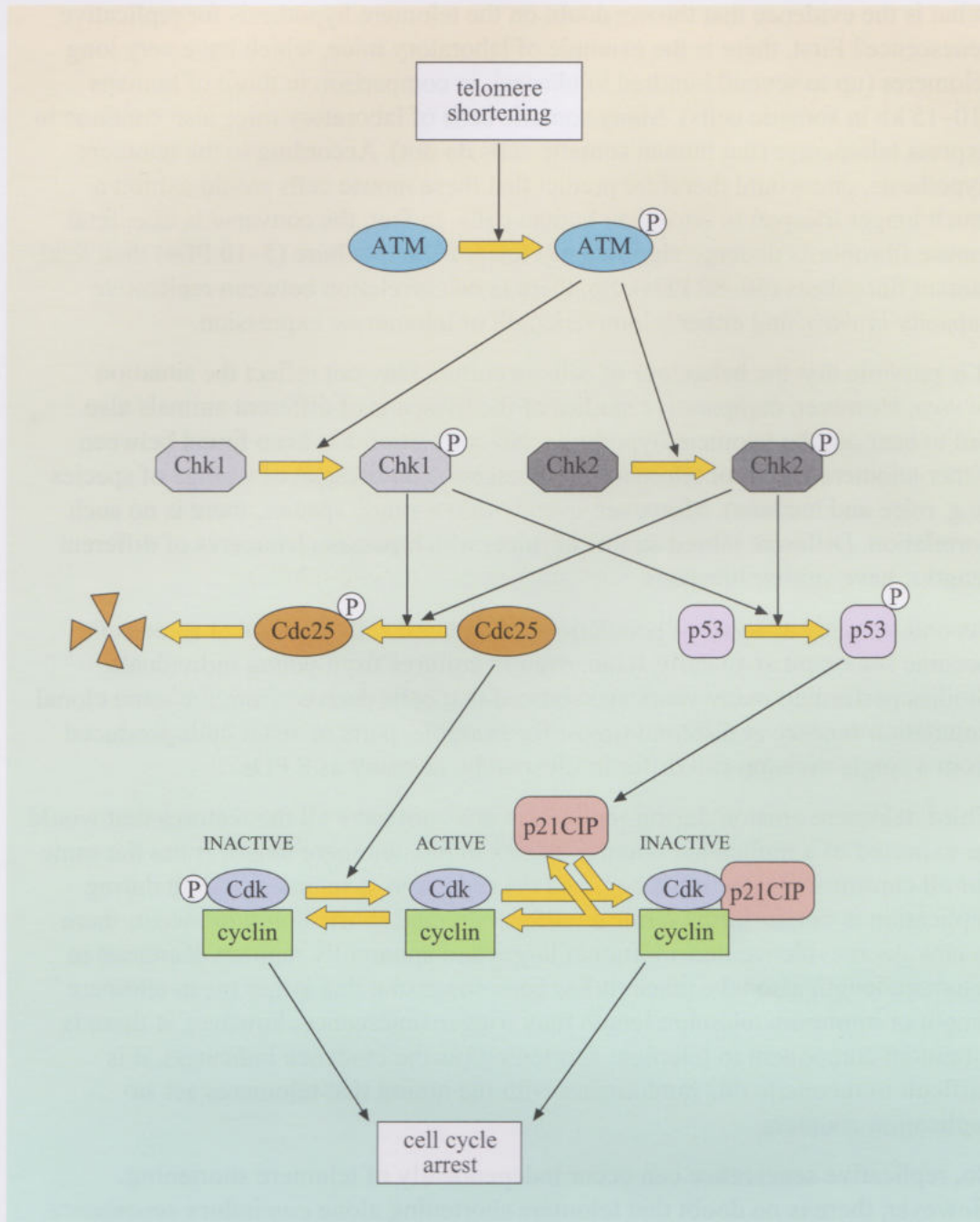


Figure 18.6

Mechanism by which detection of short telomeres by ATM induces withdrawal from the cell cycle (i.e. cell cycle arrest). Telomere shortening causes phosphorylation of ATM, which in turn causes phosphorylation of Chk1 and Chk2. Phosphorylated Chk1 and 2 cause phosphorylation of Cdc25, which results in its breakdown, and the activation (via phosphorylation) of p53, which in turn results in inactivation of the cyclin–Cdk complex. (Note that this figure is similar to Figure 8.27.)

- ☐ How does p53 activation cause apoptosis?
- ☒ By activating transcription of pro-apoptotic Bcl-2 family proteins, such as Bax (Chapter 14).

The telomere hypothesis was supported by the observations that transfection of the gene encoding the catalytic subunit of telomerase into many (but not all) cell types resulted in their *immortalization*, i.e. they no longer became senescent in culture but continued to divide. More recent evidence, however, suggests that although telomere shortening is important in triggering senescence in human cells, it is not the *only* mechanism, nor is it likely to be the mechanism that triggers senescence in rodent cells. Thus the generality of the original telomere hypothesis has been disputed.

What is the evidence that throws doubt on the telomere hypothesis for replicative senescence? First, there is the example of laboratory mice, which have very long telomeres (up to several hundred kilobases), in comparison to those of humans (10–15 kb in somatic cells). Many somatic cells of laboratory mice also continue to express telomerase (but human somatic cells do not). According to the telomere hypothesis, one would therefore predict that these mouse cells would exhibit a much longer lifespan *in vitro* than human cells. In fact, the converse is true; fetal mouse fibroblasts undergo significantly fewer PDs in culture (5–10 PDs) than fetal human fibroblasts (50–80 PDs). So, there is no correlation between replicative capacity *in vitro*, and either telomere length or telomerase expression.

It is possible that the behaviour of cells in culture may not reflect the situation *in vivo*. However, comparative studies of the lifespans of different animals also fail to bear out the telomere hypothesis. No correlation has been found between either telomere length or telomerase expression, and lifespan of a range of species (e.g. mice and humans). Moreover, even within a single species, there is no such correlation. Different inbred strains of mice, which possess telomeres of different lengths, have similar lifespans.

Second, detailed analysis of populations of cultured cells shows that some cells become senescent at an early stage, even in cultures from young individuals. Studies performed many years ago showed that cells derived from the same clonal population senesce at different times; for example, pairs of sister cells produced from a single division can differ in lifespan by as many as 8 PDs.

Third, telomere erosion during replication does not have all the features that would be expected of a replication counter. For example, telomere length is not the same for all chromosomes within a cell, and the reduction in telomere length during replication is not uniform. While a uniform gradual shortening does occur, there is now good evidence that additional larger and apparently random decreases in telomere length also take place. It has been suggested that either mean telomere length or minimum telomere length may trigger senescence; however, if there is a random component to telomere shortening (as the evidence indicates), it is difficult to reconcile this randomness with the notion that telomeres act as replication counters.

So, replicative senescence can occur independently of telomere shortening. However, there is no doubt that telomere shortening alone *can* induce senescence. So how can these disparate data be reconciled? A likely explanation emerges from experiments showing that *telomere-dependent* senescence can be triggered *before* telomeres reach a critically short length. To understand these experiments, we need to look more closely at the structure of the telomere.

Changes in telomere structure

Telomeres consist of double-stranded DNA, several thousand base pairs long. At the end of the telomere is an overhang of the 3', G-rich strand, which is about 200–300 bp in length. This single-strand overhang is the substrate for telomerase. Evidence indicates that the end of the telomere forms a terminal loop, and that the overhang region becomes associated with the double-stranded telomeric DNA. Specific proteins, including TRF1, TRF2 and Pot1, bind to the loop, as shown in Figure 18.7. These proteins stabilize the loop. The complex (sometimes known as a 'cap') is crucial for chromosome stability, since exposed telomere ends would otherwise be detected as DNA breaks, leading to activation of the repair system, recombination and chromosome fusion (see Chapter 9, Section 9.5.2).

All cells originating from an individual cell by division constitute a clonal population, or clone.

- ☐ What must happen to the terminal loop at each round of replication?
- ☒ It must unfold and then reform.

Unfolding of the terminal loop at replication exposes the ends of the chromosomes, so that they are vulnerable, as described above. Loop formation has been shown to be dependent in particular on TRF2; if TRF2 function is disrupted, the cell cycle is arrested, by activation of ATM and p53, or apoptosis may be triggered. Studies in which TRF2 was overexpressed in a human fibroblast cell line showed that the protein had a protective effect, delaying senescence and allowing cells to continue proliferation, even when the length of their telomeres was critically short. Evidence suggests that it is the *structure* of the end of the telomere that determines whether or not telomere-dependent senescence is triggered. Either the loop itself, or possibly the other components of the terminal complex of proteins (binding of which may be facilitated by TRF2) could be involved.

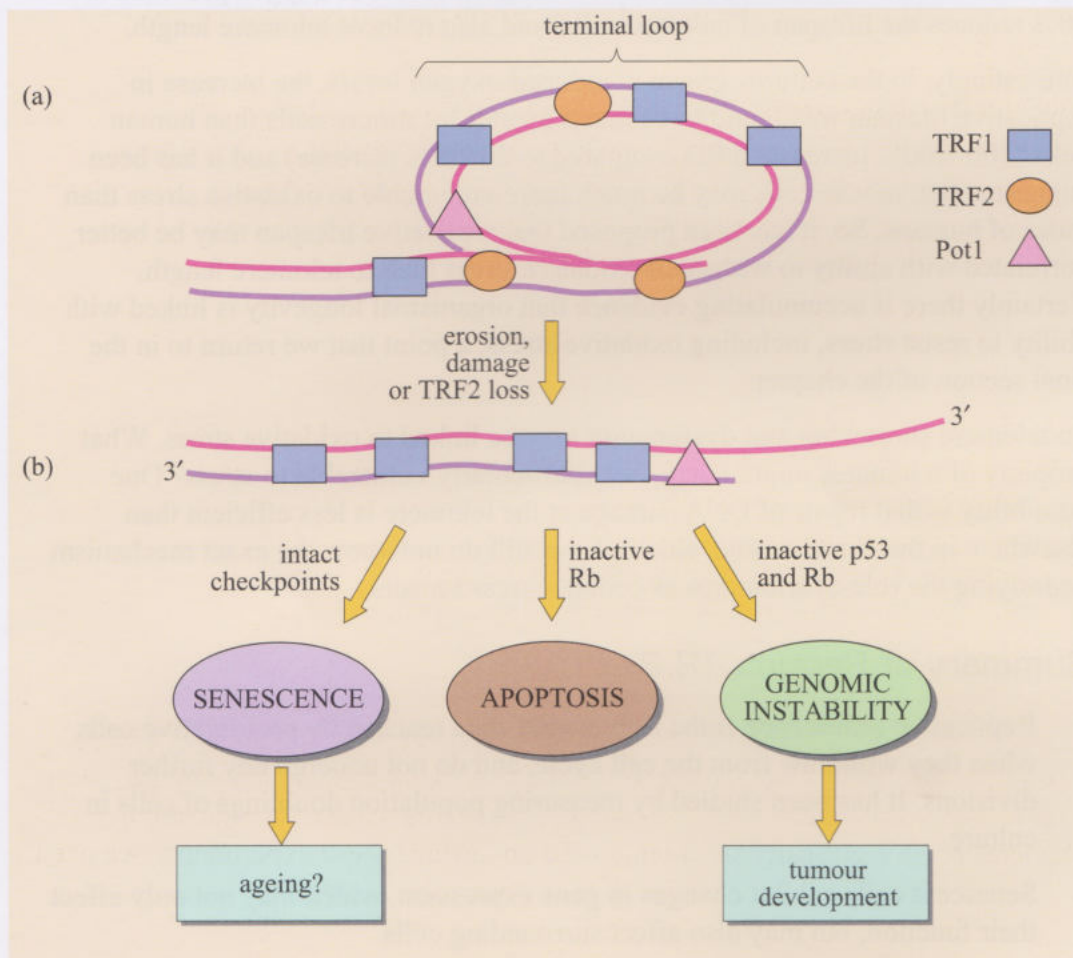


Figure 18.7 (a) The structure of a telomere, showing the terminal loop, and some of the proteins that associate with it. At the end of the telomere is an overhang of the 3' strand. This region forms a loop that, together with a number of proteins, including TRF1, TRF2 and Pot1, protects the free ends of the telomere. (b) Disruption of the loop, e.g. by erosion or damage or reduced expression of TRF2, leads to senescence, apoptosis or genomic instability, depending on the cell cycle checkpoints present in the cells. Cells with intact checkpoints (both p53 and Rb active) enter senescence, resulting in tissue ageing. If Rb is inactive, and only p53 is active, cells tend to undergo apoptosis. If both checkpoints are disrupted (neither p53 nor Rb are active), proliferation can continue, leading to genomic instability (and possibly tumour formation).

Do telomeres measure stress and damage accumulation?

An alternative proposal is that telomeres do not act as replication counters, but as 'sentinels' that monitor and respond to cellular stresses, notably oxidative stress. The argument is that oxidative DNA damage occurs preferentially at the telomeres, leading to increased shortening under conditions of stress, or to an uncapping or change of state of the telomere, such that ATM and subsequently p53 and its downstream pathway are activated and senescence ensues. What is the evidence for this hypothesis?

Data from cultures of a variety of cell types grown in different levels of oxygen provide correlative evidence for the role of telomeres as monitors of oxidative stress. Cells are exposed to much higher levels of oxygen when in culture (the ambient O₂ concentration is 20%) than their counterparts *in vivo* (2–5% O₂). Lowering the concentration of oxygen provided to cultured cells (to 2–3%), increases the replicative lifespan of both mouse and human cells. The converse experiments have also been performed; raising the levels of oxygen provided to 40% reduces the lifespan of cultured cells, and also reduces telomere length.

Interestingly, in the cultures grown at reduced oxygen levels, the increase in replicative lifespan was found to be much greater for mouse cells than human cells (100–200% increase in PD compared to 20–30% increase) and it has been suggested that mouse cells may be much more vulnerable to oxidative stress than those of humans. So, it has been proposed that replicative lifespan may be better correlated with ability to withstand oxidative stress than to telomere length. Certainly there is accumulating evidence that organismal longevity is linked with ability to resist stress, including oxidative stress, a point that we return to in the final section of the chapter.

So telomere shortening and dysfunction may be linked to oxidative stress. What property of telomeres might make them particularly vulnerable to stress? One possibility is that repair of DNA damage at the telomere is less efficient than elsewhere in the chromosome, although we still do not know the exact mechanism underlying the role of telomeres as cellular stress sensors.

Summary of Section 18.3

- 1 Replicative senescence is the irreversible state reached by proliferative cells when they withdraw from the cell cycle, and do not undergo any further divisions. It has been studied by measuring population doublings of cells in culture.
- 2 Senescent cells exhibit changes in gene expression, which may not only affect their function, but may also affect surrounding cells.
- 3 Several different events can cause cells to become senescent; these include telomere shortening, some types of DNA damage, decondensation of chromatin, overactivity of some mitogenic stimuli and activation of some oncogenes.
- 4 Telomeres may be preferentially susceptible to DNA damage, and therefore not only trigger replicative senescence due to shortening caused by repeated cell division, but also act as a type of 'sensor' of DNA damaging events, such as oxidative stress.

18.4 Ageing of post-mitotic cells

Post-mitotic cells such as neurons and skeletal muscle cells are a major component of adult mammals, and in some model organisms, *all* adult cells apart from germ cells are post-mitotic.

- ☐ In which model organisms are all the somatic cells post-mitotic in the adult?
- ☒ *C. elegans* and *D. melanogaster* (Section 18.1).
- ☐ In mammals, do post-mitotic cells in organs such as the brain and skeletal muscle occur in isolation from proliferative cells?
- ☒ No, even in tissues that have large numbers of post-mitotic cells, proliferative cells are present. An example is the brain, where some types of glial cells are able to proliferate, and do so, especially after brain injury. Skeletal muscle has associated connective tissue and although these connective tissue cells turn over very slowly, they are nevertheless capable of dividing.

Post-mitotic cells exhibit a number of changes during ageing. Major features of cellular ageing that post-mitotic cells are likely to show are the accumulation of nuclear mutations, mitochondrial damage (including mutations of mitochondrial DNA) and protein modifications that affect protein processing as well as transcriptional regulation. Here we briefly consider these aspects of ageing, focusing particularly on evidence for mitochondrial damage and alterations in protein processing.

18.4.1 Mitochondrial damage

There is now a considerable body of evidence supporting the involvement of mitochondria in ageing. Results from many experiments suggest that mitochondrial function is impaired with age, and that this impairment may lead to cellular, tissue and organismal ageing.

Age-associated changes in mitochondria are particularly noticeable in post-mitotic cells. Increased numbers of enlarged mitochondria are present and mitochondrial membrane potential (see Chapter 4, Figure 4.10) declines; there is a decline in the activity of the TCA cycle, impaired generation of energy and an increase in the production of ROS. Electron microscopy has shown that mitochondria become swollen, cristae may be lost and, in some cases, there may be complete disruption of mitochondrial organization.

- ☐ Suggest a mechanism by which mitochondria could be damaged, and explain the reason for your suggestion.
- ☒ Oxidative damage; mitochondria are the site of oxidative phosphorylation, and therefore exposed to high levels of free radicals.

Although all components of mitochondria, including proteins, membranes and mitochondrial DNA, are vulnerable to oxidative damage, damage to mtDNA is by far the most significant in terms of cellular function, for several reasons.

First, protein products encoded by mtDNA are essential to respiration. Recall that the TCA cycle and the electron transport chain both take place in the mitochondria.

These processes involve a large number of enzymes and carrier proteins. Most of these proteins are encoded by the nuclear genome, synthesized in the cytosol and imported into the mitochondria. However, 13 components of the electron transport chain are encoded by the mitochondrial genome. (The mitochondrial genome also encodes two ribosomal RNAs and 22 transfer RNAs used for mitochondrial protein synthesis.) Thus, deleterious mutations in the mitochondrial genome can have serious consequences for energy generation by the cell.

Second, mitochondrial genes are more vulnerable to damage than nuclear genes. This is because, in addition to the location of mitochondrial DNA:

- mtDNA is not complexed with histones, which in nuclear chromatin, offer some protection against oxidative damage;
- almost all mtDNA is coding DNA, so any mutations that occur may affect gene products;
- DNA repair is not as efficient in the mitochondria, as not all DNA repair mechanisms take place.

Third, mutations in mtDNA are associated with a number of diseases, particularly of tissues containing post-mitotic cells, such as skeletal muscle and the nervous system. There is also good evidence that mutations in mtDNA occur during ageing. Importantly, mitochondria containing mutated DNA appear to preferentially build up in some ageing post-mitotic cells, by an as yet unconfirmed mechanism. One explanation for this accumulation is that autophagy (Section 12.6.4) of damaged mitochondria is impaired in ageing cells, possibly because of the oxidative damage to their proteins and membrane lipids.

Mutations in mtDNA in ageing neurons and muscle can be demonstrated histochemically. Among the respiratory chain proteins encoded by the mitochondrial genome are some of the subunits of the cytochrome oxidase complex. This complex (also known as complex IV) is composed of 13 different polypeptides. The activity of the cytochrome oxidase complex can be detected by a histochemical reaction, giving a coloured reaction product. Cells that carry deletion mutations in the regions of the mitochondrial genome encoding subunits of the cytochrome oxidase complex do not stain in this reaction (i.e. they are cytochrome oxidase-negative). Such severe deletion mutations correlate with muscle atrophy and, in neurons, with neurodegenerative changes.

Recent data also indicate that the mitochondrial genome may be vulnerable in some stem cells. These data have come from analysis of the expression of mtDNA-encoded genes and nuclear-encoded genes by cells in the intestinal crypts. Recall that the epithelial cells that line the intestine have a short lifespan, and are derived from stem cells located near the bases of the crypts (Chapter 17, Section 17.4). In samples taken from human subjects, all the cells in some crypts showed abnormal expression of mtDNA-encoded subunits of cytochrome oxidase, and age-related increases in mutations in mitochondrial gene products were detected (Taylor *et al.*, 2003). An example of these data is shown in Figure 18.8.

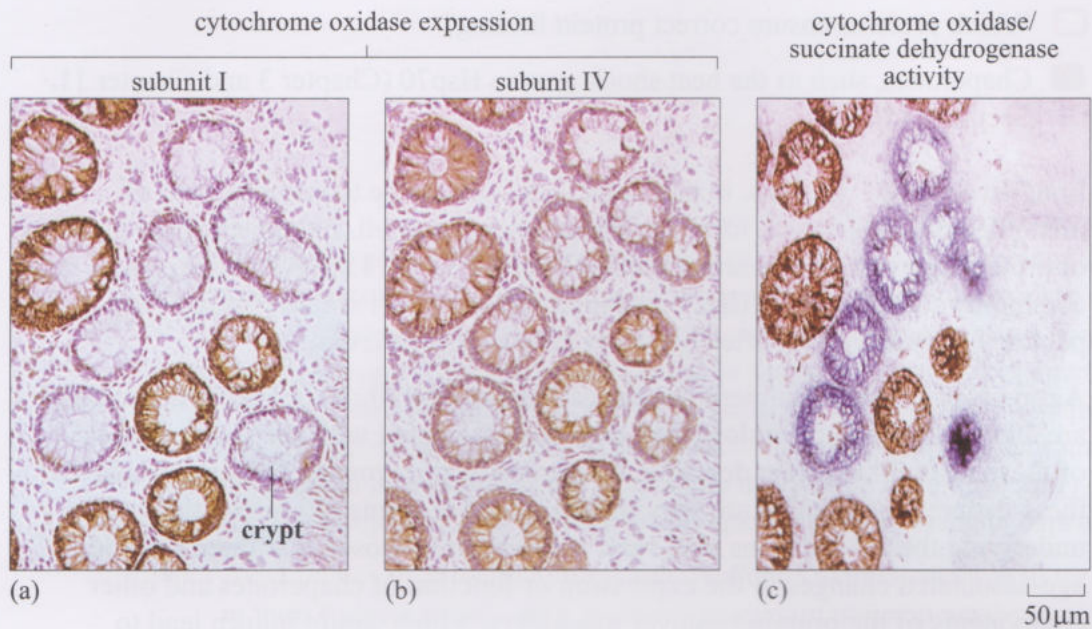


Figure 18.8 Cytochrome oxidase-negative cells in ageing human colon. Transverse sections of crypts (i.e. perpendicular to the plane of section in Figure 17.34a), showing: (a) cytochrome oxidase subunit I *expression*, revealed by immunohistochemistry, using a secondary antibody that gives a brown reaction product; (b) cytochrome oxidase subunit IV *expression*, also shown by immunohistochemistry, and again giving a brown reaction product; (c) dual cytochrome oxidase/succinate dehydrogenase *activity*, visualized by histochemical reactions with different colour-reaction products. Subunit I of cytochrome oxidase is encoded by mtDNA, whereas subunit IV and succinate dehydrogenase are encoded by nuclear DNA. (a) In some crypts, cells carry mutations in the mtDNA-encoded subunit I gene, resulting in loss of expression; this is detected by the absence of brown staining in some crypts. (b) The cells in these crypts continue to express subunit IV, albeit at lower levels than adjacent crypts (weaker brown staining). (c) Cytochrome oxidase activity (brown) in the subunit I-deficient crypts is lost (because they lack a component of the complex), although the activity of the nuclear-encoded mitochondrial TCA cycle enzyme, succinate dehydrogenase (blue) is retained. Note that the brown histochemical reaction of the cytochrome oxidase reaction masks the blue succinate dehydrogenase reaction in the other crypts.

18.4.2 Protein folding and accumulation

Changes in protein structure, processing and turnover have often been suggested to contribute to cellular ageing. We have already described how free radicals can damage proteins (Section 18.2.2), and you learnt how damaged proteins are usually degraded by the cell in Chapter 11.

- ☐ What are the mechanisms by which damaged proteins are degraded?
- ☒ They can be digested by lysosomes or are targeted for degradation by the proteasome (Section 11.7.1).
- ☐ How are damaged proteins tagged for degradation?
- ☒ By the addition of ubiquitin molecules, as a result of the action of ubiquitin ligases, of which there are many types that identify particular forms of damaged proteins.

In some cases, proteins that are damaged become misfolded; again there are cellular mechanisms that act to refold these proteins.

- ☐ Which proteins ensure correct protein folding?
- ☒ Chaperones, such as the heat shock protein Hsp70 (Chapter 3 and Chapter 11, Section 11.6.3).

Long-lived cells – neurons, in particular – are vulnerable to accumulation of misfolded proteins, which form aggregates within the cell. Examples of the build-up of protein aggregates in neurons were shown in Figure 11.33. Another example is commonly seen in Type II (late onset) diabetes, in which accumulation of the peptide hormone amylin often occurs in cells of the pancreas.

Aggregated proteins and peptides are called amyloid fibrils or plaques, and there are 20 or so different **amyloidoses**, diseases associated with the accumulation of these insoluble protein deposits. Interestingly, the proteins that accumulate in these different conditions are very different, and it seems likely that the causes underlying these conditions also vary. It has been proposed that there may be age-associated changes in the expression or function of chaperones and other components of the protein turnover machinery, which would in turn lead to impaired removal of damaged proteins.

18.4.3 Glycation

Glycation is the non-enzymatic glycosylation of molecules, particularly proteins. It occurs in diabetes and neurodegenerative diseases, such as Alzheimer's disease, but also during ageing. The process involves a series of reactions, started by reaction of the carbonyl group of a sugar with side-chain amino groups in peptides or proteins. The reactions lead to protein cross-linking and the formation of fluorescent pigments known as advanced glycosylation end-products (AGEs). Oxidative reactions play a role in the formation of AGEs (which are similar to the products of lipid peroxidation) such as lipofuscin (see S204, Book 3, Vol. II, Chapter 10). Long-lived proteins (i.e. those that do not turnover rapidly) are particularly susceptible; examples are extracellular matrix proteins and some intracellular proteins, including haemoglobin.

Summary of Section 18.4

- 1 Post-mitotic cells such as neurons and skeletal muscle cells exhibit a number of changes during ageing. These changes include mitochondrial damage, abnormalities in protein folding, protein accumulation, and protein glycation.
- 2 Mitochondrial function is impaired with age, and genes encoded by mtDNA are particularly vulnerable to oxidative damage.
- 3 Changes in protein folding and turnover occur with increasing age. Accumulation of misfolded or damaged proteins therefore often occurs in long-lived cells such as neurons.
- 4 Interaction of sugars with amino acid residues starts a series of reactions leading to protein glycation and the accumulation of AGEs. Long-lived proteins (e.g. extracellular matrix proteins) are particularly susceptible to damage by glycation.

18.5 Genomic instability and ageing: lessons from progeroid syndromes

Despite the existence of molecular mechanisms that detect and repair DNA damage, and trigger replicative senescence or apoptosis, some damage does take place due to some degree of genomic instability affecting both mitotic and post-mitotic cells. Genomic instability may result in accumulation of genetic damage, mutations in proteins and loss or duplication of genes. Evidence also shows that DNA damage increases with age. The possible links between DNA damage due to genomic instability and the manifestations of ageing (cellular and organismal) are, however, difficult to establish.

At the organismal level, human experimental models of cellular ageing are clearly not possible to create for ethical reasons, but the occurrence of several premature ageing syndromes has provided information about mechanisms of cellular ageing. These rare inherited syndromes are called **segmental progeroid syndromes**. They are described as 'segmental' because some, but not all, of the features of ageing are exhibited by these individuals. As stated in Section 18.1, examples of progeroid syndromes are Werner's syndrome and ataxia telangiectasia.

- ☐ Ataxia telangiectasia is a disease associated with dysfunction of which DNA damage detection system?
- ☒ Detection of double-strand breaks (Chapter 9, see Table 9.4 on p. 97).

Interestingly, in many other progeroid syndromes, the underlying mutations have also been shown to be in genes encoding proteins involved in the detection or repair of DNA damage (see Hasty *et al.*, 2003). Thus it can be inferred that DNA damage is likely to be a major cause of some aspects of normal ageing.

Not all progeroid syndromes result from dysfunction in DNA repair mechanisms, however. One syndrome, Hutchinson-Gilford progeria syndrome (HGPS), has been found to be the result of a mutation in the *lmna* gene, which encodes the nuclear A-type lamins.

- ☐ What are nuclear lamins?
- ☒ They are intermediate filaments proteins, which form a structural lattice attached to the inner face of the nuclear envelope (Chapter 8, Section 8.2.2).

Nuclear lamins have long been known to play a role in cell division (Chapter 8); more recently, evidence has also implicated a role for lamins in the regulation of gene expression. Disruption of lamins may therefore impact on cell function in a number of ways.

Summary of Section 18.5

- 1 Both mitotic and post-mitotic cells can be affected by genomic instability.
- 2 Many segmental progeroid syndromes in humans are due to mutations in genes encoding proteins that play a role in the detection of DNA damage, or DNA repair.
- 3 A mutation in the gene encoding nuclear A-type lamins has also been found in one progeroid syndrome, HGPS.

18.6 The insulin signalling pathway; ageing of cells and organisms

Some genes may be of benefit early in the life of an individual, but have detrimental effects later on, and thus could cause age-associated disease. This phenomenon is known as *antagonistic pleiotropy*.

Although 'genes for ageing' (i.e. genes encoding products that cause ageing, but have no other obvious function) have not been identified (and indeed most biologists believe such genes are unlikely to exist), evidence suggests that there are some genes whose products, in addition to conferring benefit in individuals of reproductive age and younger, may be of benefit later in life, having a protective effect against some age-associated molecular changes. The evidence for this has come from the study of *C. elegans*, and to a lesser extent, *D. melanogaster*.

You may recall from previous study (S204 Book 3, Chapter 10) that analysis of *C. elegans* mutants resulted in the identification of genes that, when mutated, extend lifespan. Several of these genes were found to encode proteins that are involved in the ability of these worms to enter a temporary state of inactivity (known as the dauer larval stage) at times when food resources are low. Some of these genes encode proteins that are components of an insulin/IGF-1-like signalling pathway (Figure 18.9).

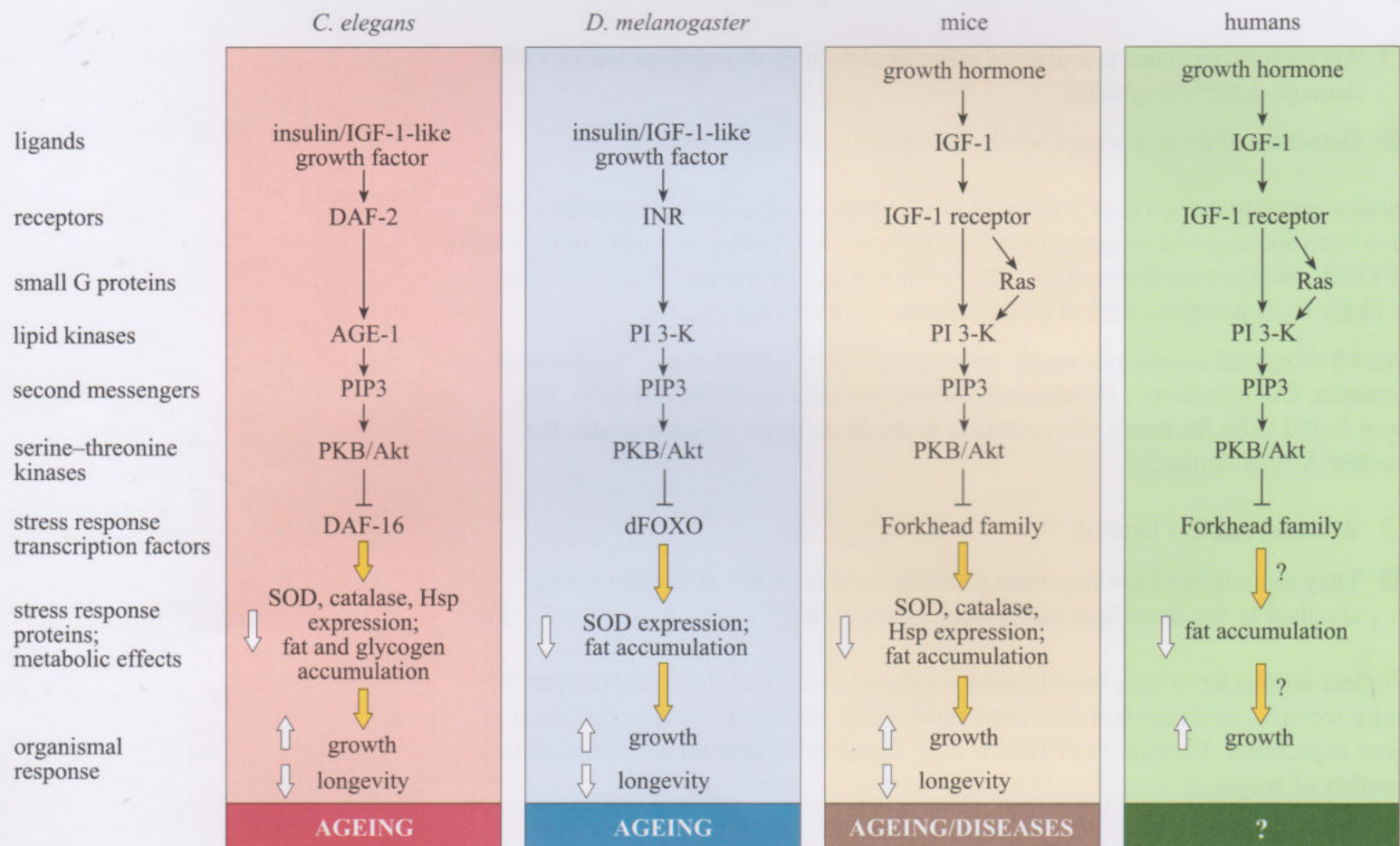


Figure 18.9 Conserved signalling pathways that regulate cellular defences and longevity. Similar pathways exist in many model organisms; *C. elegans*, *D. melanogaster*, mice and humans are shown. Studies in *C. elegans* and *D. melanogaster* show that signalling by a protein similar to insulin and IGF-1 in mammals, downregulates expression of some antioxidant enzymes and heat shock proteins, decreases fat and glycogen deposition and increases growth but decreases longevity. Key intermediates in this pathway are the transcription factors DAF-16 and dFOXO, members of the Forkhead family (Section 14.4.5). A similar pathway exists in mammals, and in mice, its activation appears to have a similar effect on stress response proteins, growth and longevity, although the details have not been fully elucidated. In humans, although mutations in the genes encoding growth hormone and IGF-1 cause growth and metabolic effects, the role of the pathway in longevity has not been established. (Note: do not confuse AGE-1, the *C. elegans* PI 3-kinase, with advanced glycosylation end-products, AGEs.)

A key component of this pathway in *C. elegans* is the transcription factor, DAF-16. Several studies have examined the genes that are downstream targets of DAF-16. Microarray analysis has shown that the transcription of several hundred genes is affected by DAF-16. Significantly, these transcriptional changes include upregulation of a number of genes encoding protective proteins, including heat shock proteins and antioxidant enzymes, but downregulation of genes encoding proteins that shorten lifespan in worms, including an insulin/IGF-1-like molecule that acts to repress DAF-16 signalling.

The effects of some of these genes on lifespan was tested by using RNA interference (Chapter 10, Box 10.5), in which worms were fed bacteria that had been engineered to contain RNA fragments corresponding to the genes of interest, to inhibit production of the corresponding protein. These experiments confirmed that a change in lifespan resulted from changes in the expression of some genes that act downstream of DAF-16. The important implication of these findings is that a number of molecules and mechanisms involved in cellular defences and metabolic processes at the cellular level affect the ageing process. They also indicate that, in the short-lived model organism *C. elegans* at least, cellular ageing is likely to be linked to lifespan. These results lend support to data that show a correlation between cellular stress resistance and lifespan of a range of species.

So, we end this chapter with the positive conclusion that, although there are no specific 'ageing genes' or 'genes for longevity', the combined actions of many genes that code for proteins regulating cellular metabolism, defence against molecular damage, and DNA repair may delay cellular ageing and also extend longevity in animals.

Summary of Section 18.6

- 1 The insulin/IGF-1-like signalling pathway in *C. elegans*, *D. melanogaster* and mice affects both stress responses and longevity.
- 2 Activation of this pathway in these three laboratory model organisms leads to downregulation of stress response genes and reduces lifespan.
- 3 Although an analogous pathway occurs in humans, and affects growth and metabolism, the effects on stress response genes and longevity are not known.

Learning outcomes for Chapter 18

When you have studied this chapter, you should be able to:

- 18.1 Define and use each of the terms printed in **bold** in the text.
- 18.2 Give a brief account of the main types of free radical, how they are generated, how they cause damage and how they are protected against.
- 18.3 Explain how replicative senescence may occur, and describe the changes that take place in senescent cells.
- 18.4 Describe briefly the age-associated changes that take place in post-mitotic cells.
- 18.5 Explain briefly how the insulin/IGF-1 signalling pathway links cellular ageing and organismal ageing in some model organisms.

Questions for Chapter 18

Question 18.1

What are the differences between superoxide and hydrogen peroxide, and what are the similarities? How are they generated and which antioxidant defences protect against damage by these substances?

Question 18.2

How do free radicals affect DNA in cells? Explain the result of the action of free radicals on DNA in replicative cells. Give an example of an effect of DNA damage in post-mitotic cells.

Question 18.3

What is DAF-16? What would be the effect of a mutation in the *daf-16* gene that increased DAF-16 activity, on antioxidant enzymes and longevity in *C. elegans*?

References

- Bird, J., Ostler, E. L. and Faragher, R. G. A. (2003) Can we say that senescent cells cause ageing?, *Experimental Gerontology*, **38**, pp. 1319–1326.
- Campisi, J. (2001) From cells to organisms: can we learn about aging from cells in culture? *Experimental Gerontology*, **36**, pp. 607–618.
- Halliwell, B. and Gutteridge, J. M. C. (2000) *Free Radicals in Biology and Medicine*, Oxford Science, Oxford.
- Kirkwood, T. B. L. (2002) Molecular gerontology, *Journal of Inherited Metabolic Disorders*, **25**, pp. 189–196.
- Kowald, A., Kirkwood, T. B. L. (1996) A network theory of ageing: the interactions of defective mitochondria, aberrant proteins, free radicals and scavengers in the ageing process, *Mutation Research*, **316**, pp. 209–236.
- Partridge, L. and Gems, D. (2002) Mechanisms of ageing: public or private?, *Nature Reviews Genetics*, **3**, pp. 165–175.
- Sampayo, J. N., Olsen, A. and Lithgow, G. J. (2003) *Aging Cell*, **2**, pp. 319–326.
- Von Zglinicki, T. (2003) Replicative senescence and the art of counting, *Experimental Gerontology*, **38**, pp. 1259–1264.

Further sources

- Hasty, P., Campisi, J., Hoeijmakers, J., van Steeg, H. and Vijg, J. (2003) Ageing and genome maintenance: lessons from the mouse?, *Science*, **299**, pp. 1355–1359.
- Gems, D. and McElwee, J. J. (2003) Microarraying mortality, *Nature (News and Views)*, **424**, pp. 259–261.
- Kirkwood, T. B. L. (2003) Genes that shape the course of ageing, *Trends in Endocrinology and Metabolism*, **14**, pp. 345–347.
- Lithgow, G. J. and Gill, M. S. (2003) Cost-free longevity in mice?, *Nature (News and Views)*, **421**, pp. 125–126.

19 TUMORIGENESIS

19.1 Introduction

Tumour cells are normal somatic cells that have acquired mutations in genes for proteins that are normally responsible for regulating cell division, differentiation and survival. You have already met many of the key proteins implicated in tumorigenesis, in previous chapters, including components of the signal transduction pathways that regulate differentiation (Chapter 17), proliferation or apoptosis (Chapters 13 and 14), cell cycle control (Chapter 8) and DNA repair proteins (Chapter 9). Dysregulation of these systems in any organism causes cells to proliferate out of control, and in multicellular organisms this may result in the formation of tumours, a process known as **tumorigenesis**. In fact, much of the research in this area of basic cell biology has been fuelled by studies directed toward understanding the human disease resulting from the development of tumours, a condition known as cancer. In the process, we have learnt a great deal over the past few decades about the ways in which both normal and tumour cells operate.

In this chapter, we will discuss the set of cellular events through which a cell can progressively accumulate mutations in its genomic DNA and progress to become tumorigenic, drawing together what you have learnt about cell regulatory processes from previous S377 material. Although we will be mostly referring to human cancer, the progression of cellular changes is common to all tumours, even in plants. You will meet (or revisit) the genes and their respective proteins that are most commonly mutated, ending with the well-researched example of human colorectal cancer. We will also look at the ways in which human cancer-causing genes are identified. Throughout the chapter, we will discuss the mechanism of action of several currently used anti-cancer drugs – and the reason(s) why they are not always capable of eradicating cancer – and explore emerging cancer therapies that are potentially more effective.

19.1.1 Mutations in genomic DNA underlie tumorigenesis

The genomic DNA of all somatic cells, both dividing and non-dividing, is subject to potentially damaging chemical and physical agents which can lead to alterations in its bases. These alterations can alter the coding potential of open reading frames (ORFs), damage sequence-encoded signals (such as splice sites, promoter elements, etc.) or lead to loss of segments of DNA.

☐ Can you recall the main causes of damage to bases in DNA?

☒ These were discussed in Section 5.5. They include:

- ▶ spontaneous hydrolysis of base–sugar linkages, creating abasic sites (especially depurination);
- ▶ spontaneous deamination of cytosine;
- ▶ formation of pyrimidine dimers upon exposure to UVB light;

Most carcinogens are mutagens, i.e. they induce DNA damage. There are a few carcinogens that are not mutagens, but help 'promote' cancer, e.g. by increasing proliferation in cells previously exposed to a mutagen. These 'tumour promoters' include some viruses (see Table 19.1) and phorbol esters; the latter activate protein kinase C, thereby activating signalling pathways that promote proliferation.

- ▶ damage by reactive oxygen species (ROS), causing strand breakage and generation of 8-oxoguanine;
- ▶ addition of methyl groups by various alkylating agents;
- ▶ DNA adducts formed by bulky molecules such as tobacco-derived benzo[a]pyrene metabolites.

Such damage can therefore be spontaneous, due to the inherent chemical properties of DNA, or it can be induced by environmental mutagens. Table 19.1 shows that many of the main risk factors for human cancer relate to DNA-damaging environmental agents, and the probability of damage also increases with age. The incidence of several forms of human cancers in relation to age is shown in Figure 19.1, and the risk associated with environmental exposure to carcinogens, illustrated by exposure to cigarette smoke, is shown in Figure 19.2.

Table 19.1 Major risk factors for human cancer.

Risk factor*	Examples	Explanation
age	For many human cancers, such as colorectal cancer, risk increases with age (see Figure 19.1).	Time increases the probability of both spontaneous and environmentally induced DNA damage in somatic cells.
environmental chemical carcinogens	1 Benzo[a]pyrene metabolites from tobacco smoke (see Figure 19.2). 2 Aflatoxin, from a fungus that may contaminate peanuts; the most potent carcinogen known causing liver cancer.	Mutagens bind to, or otherwise damage, DNA. Both benzo(a)pyrene and aflatoxin metabolites form DNA adducts with guanine.
radiation	UVB light, X-rays	Radiation damages DNA both directly, e.g. by inducing direct breaks, and indirectly by elevating ROS levels in the cell.
genetic predisposition	1 Familial breast cancer, mostly associated with inherited mutations in the <i>BRCA1</i> or <i>BRCA2</i> genes (see Section 19.2). 2 Retinoblastoma (see Section 19.4). 3 Familial adenomatous polyposis coli (colon cancer, see Section 19.9).	Inherited mutations in genes that encode proteins critical for specific cellular functions such as DNA repair.
infection	1 Viruses, e.g. papillomaviruses (cervical cancer, see Section 19.4), Epstein–Barr virus (Burkitt's lymphoma, see Section 19.4). 2 Chronic infection/inflammation, e.g. <i>Helicobacter pylori</i> (gastric cancer), schistosomes (bladder cancer).	These DNA viruses can act as tumour promoters, by hijacking the controls on cellular proliferation. Chronic inflammation produces elevated levels of reactive nitrogen and oxygen species, together with cytokines that promote cell division and tissue regeneration. (See also Section 19.7.)

* The environmental and genetic risk factors are as relevant to individuals as they are to populations. The list is far from exhaustive, but illustrates the link between risk factors and cellular mechanisms described in the text.

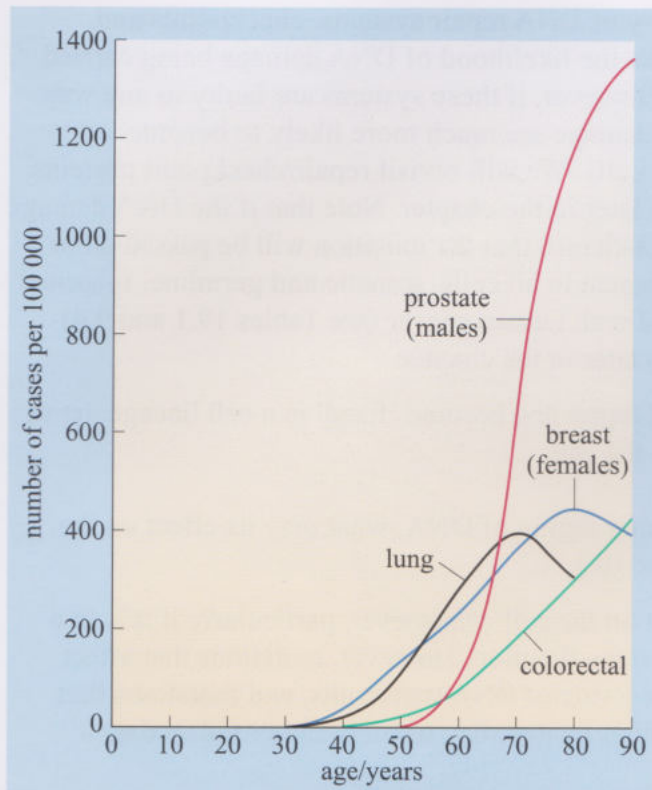


Figure 19.1 The incidence of the major types of human cancer increases with age.

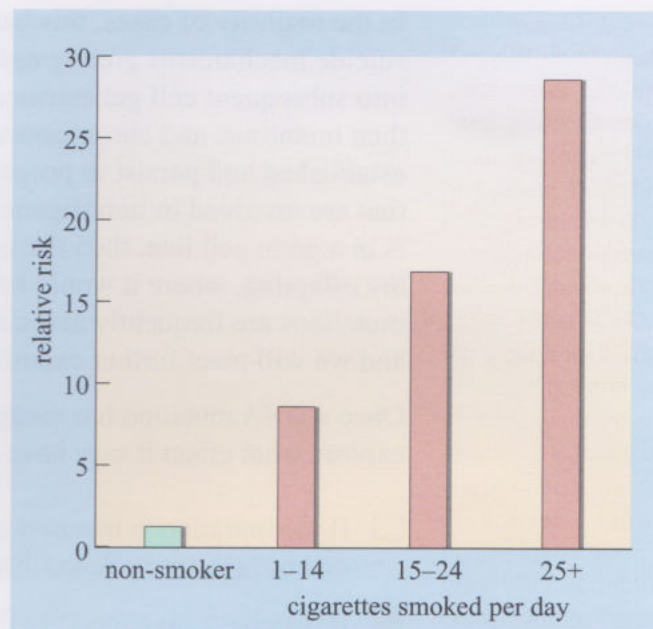


Figure 19.2 The relative risk of lung cancer in British males increases dramatically with the number of cigarettes smoked per day. Cigarette smoke contains over 50 mutagens and carcinogens, including benzo[a]pyrene and its metabolites. (The interaction between benzo[a]pyrene and DNA is shown in Figure 5.21.) The relative risk of a disease (e.g. lung cancer) in a population is the ratio of the incidence rate of the disease among individuals exposed to a risk factor (e.g. cigarette smoking) over a defined period of time to the incidence rate of the same disease among unexposed individuals.

☐ How is DNA damage normally repaired?

☒ The mechanisms were discussed in Section 9.8 and include the following:

- ▶ Certain base modifications can be directly repaired by specialized repair proteins.
- ▶ Other single-base damage may be repaired by base excision repair.
- ▶ Larger lesions that distort the helix can be detected and repaired by the process of nucleotide excision repair.
- ▶ Mismatch repair is used for mismatched pairs, insertions and deletions.
- ▶ Where there is no complementary strand template, copy repair can use the sister chromatid (dividing cells) or a homologous chromosome (non-dividing cells) as a template.
- ▶ Double-strand breaks are either repaired by direct end-joining or by copy repair.

☐ When DNA damage arises, how does a normal mammalian cell respond? (Think back to Chapters 8, 9 and 14.)

☒ First by monitoring the integrity of the genome before committing itself to division (the G1/S checkpoint). The kinases ATM and ATR are activated by double-strand breaks and blocked DNA replication (Figure 8.27). They activate Chk1 and Chk2, leading to cell cycle arrest (via Cdc25 and p53), allowing time for repair before replication. If suitable repair is not achieved, chronic activation of ATM/ATR triggers apoptosis of the DNA-damaged cell (via p53).

In the majority of cases, this battery of DNA repair systems, checkpoints and suicide mechanisms greatly reduces the likelihood of DNA damage being carried into subsequent cell generations. However, if these systems are faulty in any way, then mutations and chromosomal damage are much more likely to become established and persist in progeny cells. We will revisit repair/checkpoint proteins that are involved in tumorigenesis later in the chapter. Note that if the DNA damage is in a germ cell line, then there is a chance that the mutation will be passed on to the offspring, where it would be present in all cells, somatic and germline. Inherited mutations are frequently associated with human cancer (see Tables 19.1 and 9.4) and we will meet further examples later in the chapter.

Once a DNA mutation has escaped repair and become 'fixed' in a cell lineage, let us explore what effect it may have on the cell.

- ☐ If the mutation is in a *non-coding* region of DNA, what may its effect on the cell be? (Refer back to Chapter 10.)
- ☒ It may have no apparent effect on the cell whatsoever, particularly if it is in a region of DNA that has no obvious function. However, mutations that affect splice sites may affect the processing of RNA transcripts, and mutations that affect target sites for transcription factors may result in either reduced or elevated gene expression.
- ☐ If the mutation is in a *coding* region of a DNA, what may its effect on the cell be? (Think back to Chapters 3 and 11.)
- ☒ It may have no effect at all, if the mutation does not change the resulting amino acid sequence or if an amino acid change does not modify the function of the encoded polypeptide. Alternatively, a mutation may alter the function of the encoded polypeptide by producing a change in its primary structure, or it may affect its translation by generating a premature stop codon (e.g. *BRCA1*) or by removing the correct stop codon.

A **silent mutation** is one that either does not result in a change of amino acid (because the mutated codon still encodes the same amino acid) or produces a change of amino acid that does not modify the function of the encoded polypeptide. Silent mutations are of little consequence, and they are most often found in non-conserved regions of the protein, which are less likely to be critical for protein function. However, DNA mutations may result in an amino acid change that alters the structure and function of the protein in many ways, but resulting primarily either in an increase or decrease in the activity of the encoded protein, or it may result in a change in its level of expression. For example, insertions, deletions or mutations may cause the appearance of a premature stop codon.

- ☐ What are the possible consequences of the generation of a premature stop codon, and how will this affect the expression of the protein?
- ☒ The RNA may be degraded by nonsense-mediated decay (see Section 11.4.6), resulting in reduced expression of the protein. If the protein is translated, it is likely to have an increase or a decrease in its functional activity.

Chromosomal damage may also result in altered gene expression and/or activity of the encoded protein. For example, chromosomal translocations can result in joining together of sections of two different proteins, producing either a non-functional

protein product or a newly functional hybrid protein with aberrant activity. In other cases, genes can be positioned into different transcriptional environments that influence gene expression, such as near enhancers or silencers.

Most DNA mutations do *not* lead to cancer – many mutations are silent, and many others cause malfunctioning of the protein product that will lead to the ageing or death of the cell. However, certain genes can acquire mutations that cause the cell to persist or proliferate irrespective of normal regulatory events. These genes are collectively termed **cancer-critical genes** and we will examine the most important of them in detail later in the chapter. For now, though, let us establish what kind of genes these are and what their modes of action tend to be.

- ☐ What is the likely effect on a cell of a mutation that increases the activity of a protein kinase involved in signal transduction pathways triggered by growth factors?
- ☒ If the protein kinase controls an activation step in a growth factor signalling pathway it would lead to activation of the downstream components of the pathway, even in the absence of a growth factor. This would lead to an increased rate of cell proliferation.

Such mutated genes have what is called a *gain of function*. The mutated genes are called **oncogenes**, and their non-mutated versions are called **proto-oncogenes**. Oncogenes tend to be involved in growth-inducing and survival pathways, and include receptors, protein kinases, small G proteins and transcription factors. The archetypal example of a proto-oncogene is *ras* – it has mutations in about 25% of known human tumours, which result in the expression of hyperactive forms of Ras. This increased Ras activity causes the signal transduction pathways downstream of the Ras protein to be abnormally activated, usually leading to more cell division.

As well as genes that promote cancer by a gain of function, the opposite scenario also occurs. Certain genes are implicated in human cancer if they sustain a *loss of function*, through the acquisition of inactivating mutations or by deletion of the gene.

- ☐ What kind of functions are such genes likely to have under normal circumstances?
- ☒ They are likely to serve as brakes on cell division, or be part of growth-inhibitory, apoptosis or differentiation signalling pathways.
- ☐ You met one example of a cancer-critical gene in Chapter 8 that operates on cell cycle checkpoints. Can you recall what it is, and its function?
- ☒ The *Rb* gene product, Rb, serves as a brake on cell division (see Section 8.3.4).

We will learn more about *Rb* later in the chapter. Cancer-critical genes whose products work by suppressing growth and/or survival may also be associated with tumour development, through loss-of-function mutations. These genes are called **tumour suppressor** genes.

The acquisition of cellular properties such as increased cell proliferation or increased survival or failure to differentiate, gives a cell a competitive advantage over the surrounding cells. Indeed, tumorigenesis can be described as a kind of **microevolutionary process**. Most mutations will be disadvantageous to a cell and

the cell may not survive to pass on its mutations, but some mutations give cells competitive proliferative or survival advantages.

- ☐ What will happen to a cell that acquires a competitive proliferative advantage?
- ☒ It will proliferate more than its neighbours and all its progeny will carry the same advantageous mutation(s).

This can, in a way, be seen as a form of natural selection at the cellular level (this argument will be developed further in Sections 19.2 and 19.6).

Some mutations in certain genes may even predispose the cell to further genetic change, resulting in a state of genetic instability.

- ☐ Can you suggest what groups of genes may predispose to genetic instability if mutated?
- ☒ Genes for DNA repair proteins and for proteins that check the state of DNA before allowing the cell to replicate (some examples are in Table 9.4).

Mutations in genes involved in DNA repair or in cell cycle checkpoints tend to accelerate the cellular evolution process considerably. Genetic instability results in many microscopically invisible mutations, and is a common feature of most tumours. In addition, the karyotype in advanced tumours may become abnormal by chromosome rearrangement or by aneuploidy.

The karyotype of a cell describes the number and structure of its chromosomes.

A single critical mutation is not enough to convert a cell into the completely unregulated proliferative state we know as cancer. However, the first critical mutation may increase survival time or increase proliferation just enough to give more opportunity for a second critical mutation to occur within that expanded clone of cells (see Figure 19.3). Again, cells harbouring advantageous mutations for proliferation will divide more rapidly and their cellular progeny will prevail over those cells that have either deleterious mutations or no mutations. Several successive rounds of acquisition of critical mutations in different genes in different regulatory pathways of dividing cells are usually needed for tumours to develop.

Although tumours usually have a clonal origin (i.e. one cell harbouring a specific advantageous mutation gives rise to all the progeny), as it becomes more advanced and especially as mutations leading to genetic instability are acquired, the cell population becomes more heterogeneous; that is, within the same tumour there may coexist many different cell 'clones' with different multiple mutations but all originating from a single abnormal tumorigenic cell (as seen in Figure 19.3), each clone with its own particular growth advantages.

19.1.2 Stages and classification of human tumours

Before we explore the cellular and molecular mechanisms that underlie tumorigenesis, we will introduce the clinical terminology of human cancer, which provides a framework for understanding the cell biology.

As mutations accumulate within the tumour cell population, not only does the tumour grow, but also the morphology and behaviour of the cells within the tumour change. Pathologists have developed well-defined classifications for the different stages of tumour progression, based upon these cellular changes. The classification of tumours is usually determined by their histological and anatomical characteristics.

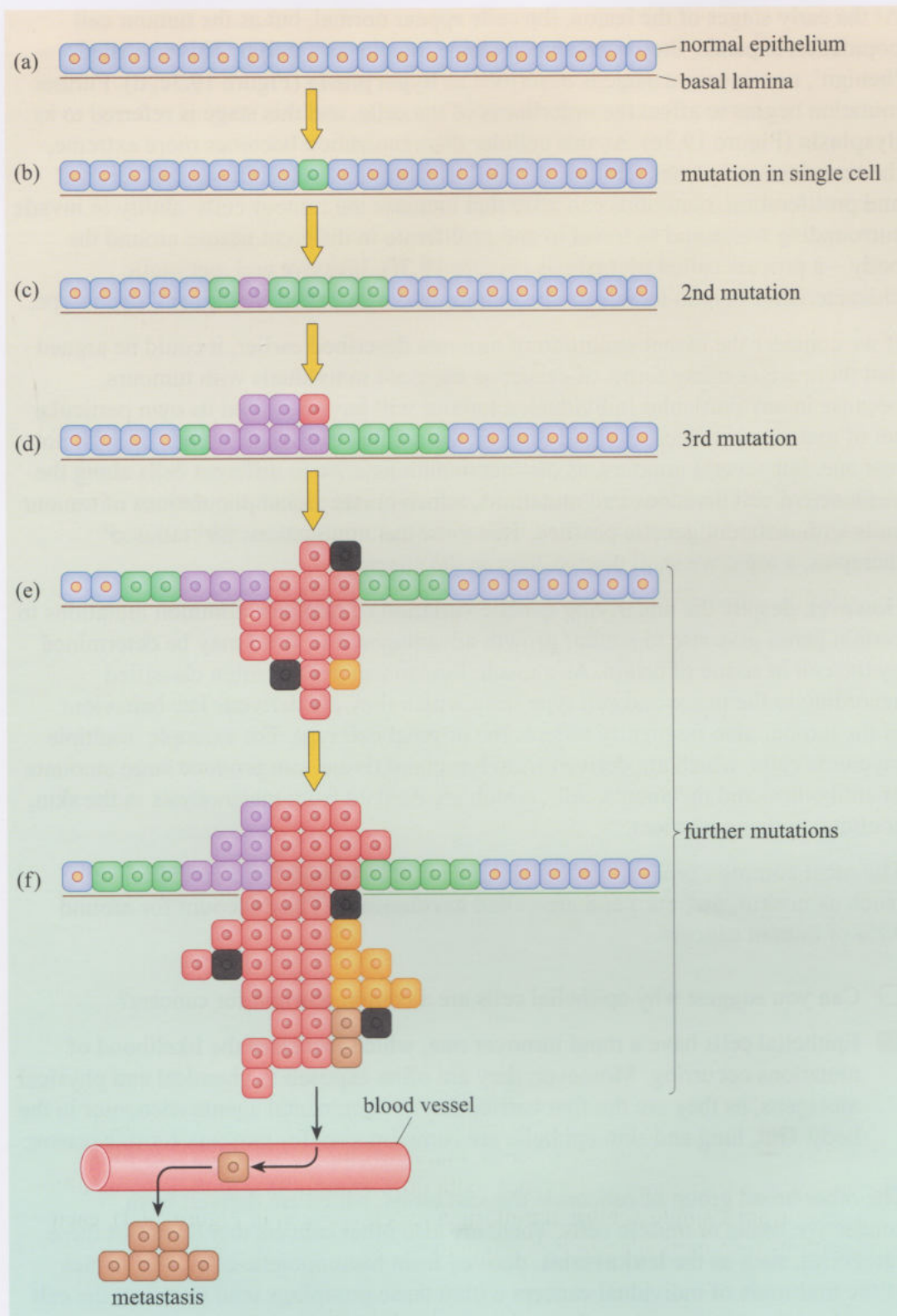


Figure 19.3 The clonal evolution of a tumour. (a) A mutation in a single cell gives rise to increased cell proliferation. (b–d) Cells with further mutations may acquire other growth advantage characteristics and their progeny become the dominant cell clone in the tumour (purple in d). (e, f) Mutations may also lead to genetic instability, enhancing the probability of DNA mutations, which can either lead to growth advantage (red) or cell death (black). Successive rounds of cell division and gene mutation lead to the formation of different cell clones with multiple different mutations (yellow, brown and black cells), which may result in even more ‘aggressive’ growth properties such as metastatic capacity (brown; see Section 19.1.2) or in clonal extinction (black).

At the early stages of the lesion, the cells appear normal, but as the tumour cell population expands, they become detectable, maybe as a **polyp**. This so-called 'benign', non-invasive stage is described as **hyperplasia** (Figure 19.3c, d). Further mutation begins to affect the orderliness of the cells, and this stage is referred to as **dysplasia** (Figure 19.3e). As this cellular disorganization becomes more extreme, the term 'cancer' comes into use. As well as mutations that enhance cell survival and proliferation, mutations can arise that increase the tumour cells' ability to invade surrounding tissue and to travel to and proliferate in different tissues around the body – a process called **metastasis** (Figure 19.3f). Invasive and metastatic characteristics help to transform the tumour into a so-called 'malignant' phenotype.

If we consider the clonal evolution of tumours described earlier, it could be argued that there are as many forms of cancer as there are individuals with tumours, because in any particular individual, a tumour will have acquired its own particular set of mutations. Indeed, it could even be argued that each patient ends up with not just one, but several tumours, as distinct mutations arise in different cells along the sequence of cell divisions and mutations, which create clonal populations of tumour cells with different genetic profiles. This view has implications for 'tailored' therapies, a topic we shall discuss later in the chapter.

However, despite the underlying genetic variation of tumours, common mutations in certain genes give rise to similar growth advantages, and these may be determined by the cell or tissue of origin. As a result, human cancers are often classified according to the tissue and cell type from which they are derived. The behaviour of the tumour also frequently reflects the original cell type. For example, multiple myeloma cells, which are derived from lymphoid tissue, can produce large amounts of antibodies, and melanoma cells, which are derived from melanocytes in the skin, continue to make pigment.

The most common types of cancer in humans are derived from epithelial cells (such as in skin, gut, etc.) and are called **carcinomas**. They account for around 90% of human cancers.

- ☐ Can you suggest why epithelial cells are a common origin for cancers?
- ☒ Epithelial cells have a rapid turnover rate, which increases the likelihood of mutations occurring. Moreover, they are often exposed to chemical and physical mutagens, as they are the first barrier that environmental agents encounter in the body. Gut, lung and skin epithelia are common sites for tumours for this reason.

The other broad group of cancers is the **sarcomas**, which are derived from connective tissue or muscle cells. There are also other cancers that do not fit these categories, such as the **leukaemias**, derived from haemopoietic cells. The names of the multitude of individual cancers within these groupings tend to reflect the cell type of origin. Also, non-invasive stages tend to have names related to the invasive version, e.g. adenoma (non-invasive) and adenocarcinoma (invasive).

19.1.3 The hallmarks of tumorigenesis

As we have discussed, tumour progression results from the growth, survival and clonal expansion of cells that have undergone successive accumulation of mutations leading to either the production of dysfunctional proteins or the loss of normal proteins. Due to the random nature of the mutational process, it is not possible to predict the order of successive mutations or which of the many cancer-critical

genes will be affected. Nevertheless, certain genetic characteristics must be acquired by tumour cells for them to progress from normal cells through hyperplasia, dysplasia to an invasive, metastatic tumour, and these characteristics are associated with mutations in genes for particular cellular functions. The main characteristics are as follows (though not necessarily in this order):

- 1 *Genetic instability.* Cells acquire mutations in genes that normally check and enable repair of DNA before cell division.
- 2 *Self-sufficiency in growth signals.* Cells acquire mutations in proto-oncogenes to produce their oncogenic versions. Oncogenes usually code for proteins involved in positively regulating signal transduction pathways in response to activation of growth factor receptors, and for transcription factors and proteins involved in cell cycle control.
- 3 *Insensitivity to growth-inhibitory signals.* Cells acquire mutations that cause inactivation of tumour suppressor genes, which frequently help control cell division and sometimes operate in the same pathways as oncogenes. Often the pathways affected are also involved in gaining genetic instability, or in avoiding apoptosis and senescence.
- 4 *Evasion of apoptosis.* Cells acquire mutations that result in the inactivation of death-related pathways, allowing them to escape apoptosis.
- 5 *Limitless replicative potential.* Cells acquire mutations that allow them to escape from terminal differentiation states and from replicative senescence.
- 6 *Sustained angiogenesis.* Cells in solid tumours can acquire mutations that enable them to establish a blood and lymphatic supply for themselves.
- 7 *Tissue invasion and metastasis.* Cells acquire mutations that enable them to invade surrounding tissue and/or colonize other tissues at distant sites. Metastasis accounts for 90% of cancer-related deaths in humans.

In the rest of this chapter, we will consider each of these properties in turn, and describe the key genetic components that, when mutated, contribute towards these characteristics.

Summary of Section 19.1

- 1 Tumorigenesis is the result of an accumulation of key mutations in cancer-critical genes in a cell's progeny, which eventually causes seriously dysregulated cell growth and survival. Exposure to environmental mutagens, failure of DNA repair mechanisms and inherited mutations contribute to the accumulation of genetic lesions.
- 2 Genes whose mutated version leads to upregulation in the protein product's activity or in its expression in tumours (usually caused by a gain-of-function mutation) are called oncogenes. In contrast, those genes whose products are downregulated in tumours (either affecting their activity or expression), usually by loss-of-function mutations, are called tumour suppressor genes.
- 3 The initial random genetic lesion occurs within a normal cell, leading to the clinical development of a benign hyperplasia. This progresses with further mutations to dysplasia, through to fully malignant cancer, which is invasive and has metastatic potential. Although clonal in origin, tumours acquire genetic instability and contain heterogeneous clones.

- 4 In medical terms, tumours are traditionally classified according to their tissue of origin. The majority of cancers in humans are carcinomas, from epithelial cells.
- 5 The key tumorigenic properties that cells acquire through successive rounds of mutation include: genetic instability, self-sufficiency in growth signals (usually by activating oncogenes), insensitivity to growth-inhibitory signals (often involving inactivation of tumour suppressor genes), avoidance of apoptosis and replicative senescence, sustained angiogenesis, and tissue invasion and metastasis.

19.2 Genetic instability

In metabolically active cells, DNA damage is not an altogether uncommon event, due to the action of normal by-products of cell respiration (e.g. free radicals) or to the action of deleterious chemical and physical agents in the external environment. DNA replication errors can also be the result of mistakes in the incorporation of bases.

- ☐ Recall from Chapter 9 the normal error rate of DNA polymerases.
- ☒ Relying on base pairing alone, the error rate would be around 1 in 10^4 bases, but because of the proofreading ability of DNA polymerases, it is reduced to around 1 in 10^7 bases.

With the presence of cell cycle checkpoint proteins that respond to the detection and repair of DNA damage, the overall rate of mutation in the eukaryotic genome is around 1 in 10^9 bases per cell division. Therefore, the normal mutation rates in an individual human cell are so low that it is highly unlikely that sufficient mutations would accumulate in a single cell line – at least within the lifetime of an individual – for the cell to acquire all the mutant characteristics that would enable it to progress to a tumour. However, the majority of tumour cells in human cancers show increased mutation rates compared to normal cells. In fact, the evolution of tumour cells accelerates massively if mutation occurs in a gene that encodes one of the proteins that detect and repair damaged DNA. Many of these genes are involved in human cancer – some of the inherited syndromes resulting from germline mutations in these genes were listed in Table 9.4.

19.2.1 DNA repair mechanisms associated with human cancer

We will now look at three key examples of genes involved in DNA repair, to show how mutations in such genes can predispose to, and/or accelerate, tumour development.

- 1 The *MLH/MSH* family of DNA mismatch repair genes in humans are homologous to bacterial *mutL* and *mutS* (Section 9.8.3). Humans with a hereditary form of colorectal cancer called hereditary non-polyposis colorectal cancer (HNPCC) have usually inherited a mutated copy of one of these genes (see also Section 19.9). The resultant compromised DNA mismatch repair mechanism would lead to progressive accumulation of single-base insertions/deletions or small repeats.

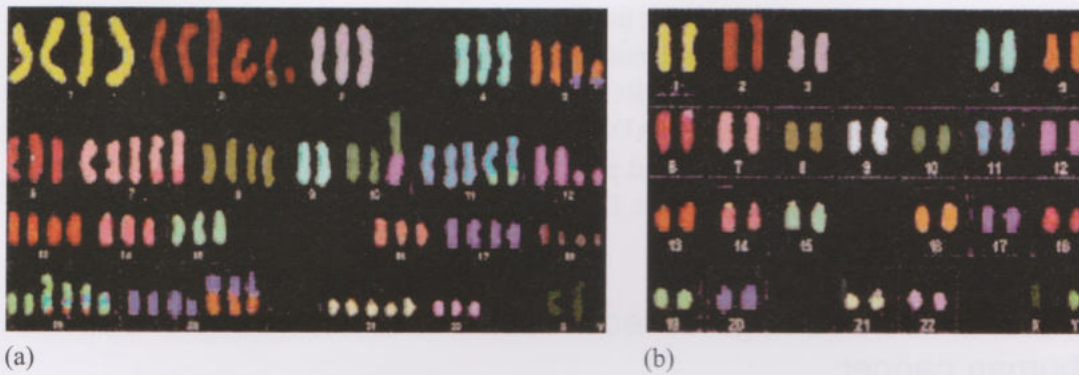


Figure 19.4 The karyotype of cells from different tumours reflects different kinds of genetic instability. Each normal chromosome is stained with probes labelled with specific combinations of fluorescent dyes, a technique known as chromosome painting. (a) The karyotype of a cell from a typical human cancer, showing gross abnormalities in the number and structure of the chromosomes (other cells from the same tumour would show variations on this karyotype). This is the kind of karyotype that would result from a loss of functional p53 or ATM (Section 19.2.2). (b) The karyotype of a cancer that has arisen as a result of defects in DNA mismatch repair, such as those affecting the MLH/MSH or the XP families of genes. The defects are generally invisible in this kind of preparation, so the karyotype appears superficially normal.

- ☐ How would such mutations be predicted to affect the karyotype of a tumour cell?
- ☒ The karyotype would be unaffected, as the cumulative genetic damage is at the DNA level and is microscopically invisible.

Figure 19.4b shows the karyotype of a tumour cell with mutations in genes involved in DNA mismatch repair compared to that of another tumour cell with different mutated genes (Figure 19.4a; Section 19.2.2).

- 2 Xeroderma pigmentosum (XP) is an inherited human disease that, amongst other clinical manifestations, confers sensitivity to UVB light, leading to around 1000-fold increased risk of developing skin cancer.
 - ☐ What kind of DNA damage does UVB radiation particularly cause?
 - ☒ Pyrimidine dimer formation (Table 5.3d, Section 5.5.1).
 - ☐ What DNA repair process usually repairs this type of damage?
 - ☒ Nucleotide excision repair (Section 9.8.1).

XP individuals show defective nucleotide excision repair which confers the vastly increased risk of skin cancer (Table 9.4). The condition may be due to mutations in one of several genes belonging to the *XP* family – named *XPA* to *XPG* – which are all implicated in nucleotide excision repair, and some of which are also associated with transcription (*XPB* and *XPD*) (Section 10.4.2).

- 3 In the 1990s, two genes were identified whose loss of function was associated with familial human breast and ovarian cancer. These tumour suppressor genes were designated *BRCA1* and *BRCA2* (from breast cancer susceptibility genes). Because there was little sequence homology with other known genes, it was not immediately apparent what function their encoded normal proteins played in the cell. At the time of writing (summer 2004), the story is still far from clear,

but it has been established that the BRCA1 protein is involved in several DNA repair mechanisms, including nucleotide excision repair and repair by homologous recombination. BRCA2 has a more limited role, regulating the activity of a protein (called RAD51) that assists with strand invasion during homologous recombination and post-replicative repair. Loss of functional *BRCA* genes would therefore decrease DNA repair and be expected to result in cumulative DNA damage.

19.2.2 DNA damage detection mechanisms associated with human cancer

ATM

Double-strand DNA breaks are detected and repaired in cells very efficiently, as they can be potentially highly mutagenic.

- ☐ How are double-strand DNA breaks normally detected in eukaryotic cells?
- ☒ ATM detects proteins bound to double-strand breaks, leading to cell cycle arrest (Figure 9.31).
- ☐ Can you predict an outcome resulting from an alteration in the function of the ATM protein by gene mutation, in terms of DNA repair?
- ☒ Double-strand DNA breaks could remain unrepaired.

As shown in Figure 9.31, double-strand DNA breaks that are not detected and repaired appropriately can trigger apoptosis. However, in some cases where cells have the ability to evade apoptotic signals, double-strand DNA breaks can result in chromosome abnormalities (Figure 19.4a) and loss of genetic information (Figure 9.29). This is seen in the human disorder known as ataxia telangiectasia (AT), a condition caused by mutations in the *ATM* gene (which stands for ataxia telangiectasia mutated). Affected individuals have reduced immunocompetence and are particularly prone to developing lymphoid tumours (these patients are also particularly sensitive to DNA damage following exposure to X-rays). The mechanism for the particular susceptibility of lymphoid cells is unclear, although it may be related to the fact that the production of antigen receptors in normal lymphocytes requires gene rearrangements that involve breaking and rejoining of the DNA of the receptor genes. Lymphoid cells of AT patients have been shown to have a high frequency of chromosomal translocations, particularly affecting the regions coding for immunoglobulin genes (B cell antigen receptor) and the T cell antigen receptor.

p53

The *p53* gene (named from the M_r of its protein product, i.e. 53 000) is mutated in almost 50% of all human tumours. The reason for its importance lies in its involvement in key areas of cell regulation.

- ☐ Can you recall the roles of p53 protein in response to DNA damage in a mammalian cell?

- p53 is involved in activation of the G1/S cell cycle checkpoint in response to DNA damage, which leads to cell cycle arrest (Figure 8.27). It also acts as an inducer of downstream pathways of DNA repair and (if repair is unsuccessful) apoptosis (Section 14.4.1), and is involved in the induction of replicative senescence (Section 18.4.3).

Intracellular p53 levels are usually elevated in response to DNA-damaging events such as hypoxia (low oxygen levels), radiation, or even excessive proliferative signals, resulting from mutated *ras* and *myc* (Section 19.3.2). As a regulator of the cell cycle, p53 acts, at least in part, by binding to DNA and inducing transcription of p21CIP which in turn binds to cyclin–Cdk complexes, thus blocking entry into and progression through S phase of the cell cycle (as described in Section 8.3.5).

- What is the role of inducing cell cycle arrest if DNA damage is detected?
- It gives the cell time to repair the DNA damage before continuing with DNA synthesis.

In this sense then, p53 inhibits growth, by indicating when internal conditions are inappropriate for DNA synthesis to proceed. If damage is very severe, then the cell will be induced to undergo apoptosis; alternatively, the cell cycle will be halted for as long as the damage remains unrepaired. This ensures that cells can only replicate if their DNA is undamaged or has undergone the necessary repairs.

- What will be the effect of losing p53 function upon a cell?
- Loss of functional p53 would allow damaged DNA to remain unrepaired as the cell cycle would not be arrested at G1/S, and mutant cells would escape apoptosis.

Chromosomal breaks and fusion between chromosomes are common in p53-deficient cells (see Figure 19.4a).

- What would the consequences be for the chromosomes of a dividing cell that contains aberrant acentric or dicentric fused chromosomes (Section 9.8.6)?
- Acentric chromosomes would be lost, as they would not attach to the mitotic spindle. Dicentric chromosomes could be pulled to both poles, creating new double-strand DNA breaks.

Successive rounds of cell division can therefore result in loss of other tumour suppressor genes or amplification of oncogenes, creating a vicious circle of genetic damage and cell dysregulation.

It is clear from these examples that there are many ways in which DNA repair and damage detection mechanisms can be disrupted, and that each type of disruption leads to a particular type of genetic chaos in the cell and its progeny. The characteristic of genetic instability in tumorigenesis is sometimes referred to as an ‘enabling’ characteristic, in that it ‘enables’ the subsequent mutation of other cancer-critical genes.

p21 is a protein also known as WAF1 or Cip1. In order to differentiate it from other proteins of similar M_r (e.g. Ras, also called p21Ras), it is referred to as p21CIP in this course, although you may come across it referred to as p21 or p21WAF in the primary literature.

Box 19.1 Therapies that target DNA in human cancer

Both radiotherapy and chemotherapeutic drugs act by damaging the DNA of dividing cells so severely that the cells undergo mitotic failure and apoptosis. For this reason, they are termed **cytotoxic** therapies. Ionizing radiation has been used in cancer therapy ever since the discovery of X-rays a century ago. Any molecule in a cell can be damaged, but damage to DNA is the most crucial in halting cell proliferation (see Figure 19.5). Table 19.2 shows the variety of mechanisms of action of ‘conventional’ chemotherapeutic agents, but these include two main functional groupings. There are those that bind directly to DNA, the *alkylating agents*. This category includes the first anticancer drug, nitrogen mustard. *Antimetabolites*, in contrast, usually act by inhibiting nucleic acid synthesis, either directly or indirectly. There are other miscellaneous agents, and a biochemically diverse collection of natural products.

The reason that these cytotoxic methods work with at least some specificity for tumour cells is because of the ability of normal cells to undergo cell cycle arrest when damaged, giving them time to repair damaged DNA, or to die in a controlled way by apoptosis. Because tumorigenic cells, in contrast, have lost the controls on cell cycle arrest, they continue to divide despite the large-scale genetic damage inflicted by cytotoxic drugs or radiotherapy, which has lethal consequences for the vast majority of tumour cells.

The clonal population of a tumour is heterogeneous and its cells are highly mutable. As a result, it is likely that some cells will become resistant to the cytotoxic treatment. The cytotoxic-treatment resistant population of tumour cells will be able to proliferate, inducing tumour regrowth, and may need to be combated with different classes of cytotoxic agents to

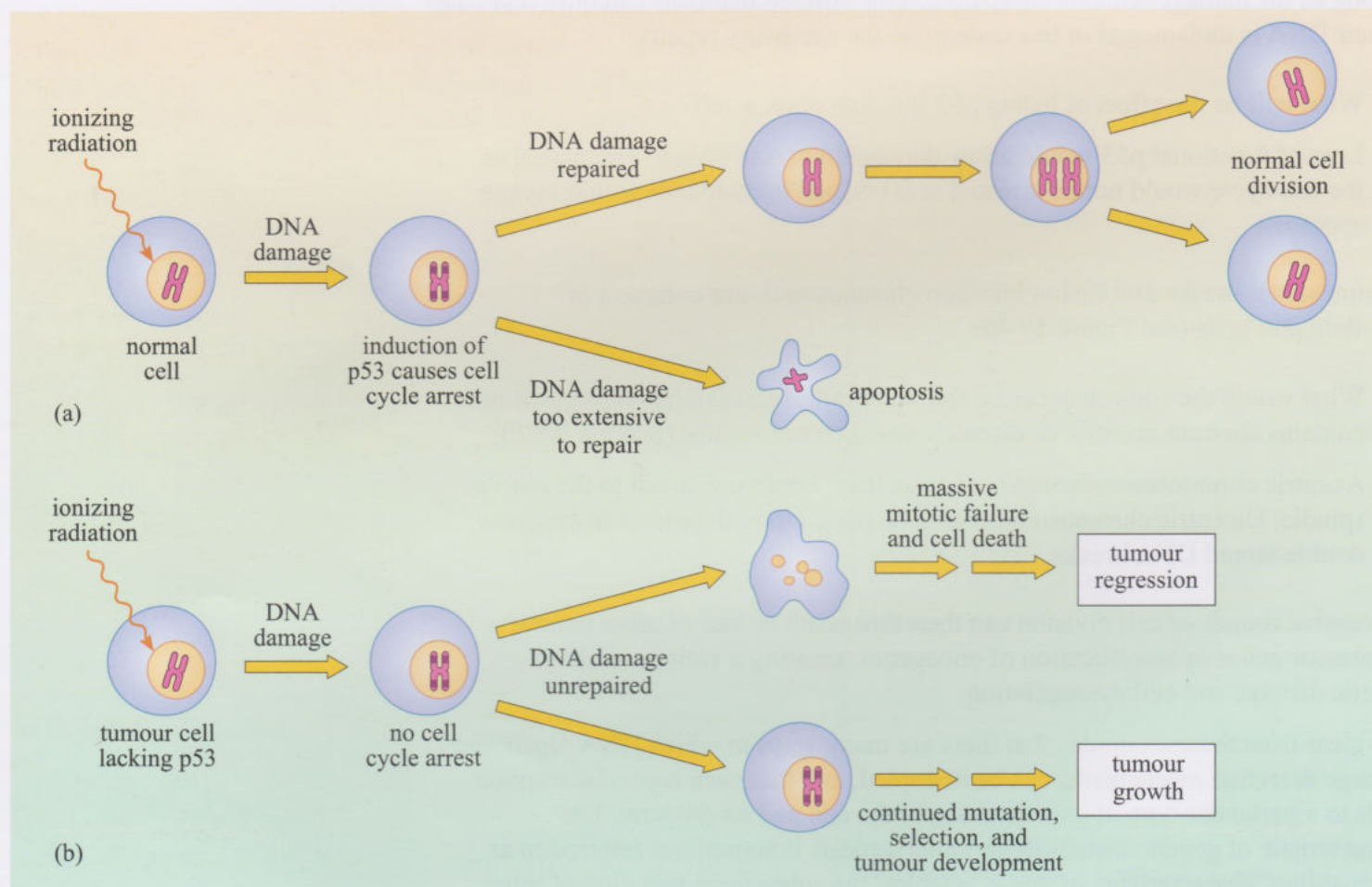


Figure 19.5 The effects of ionizing radiation on tumour versus normal cells. (a) shows that normal cells will either repair the damage, or undergo apoptosis if damage is severe. (b) shows that tumour cells, lacking normal repair mechanisms, will mostly die through mitotic failure, but if some sustain less than lethal damage, then they will proliferate (with any newly acquired mutations) and progress further towards tumour regrowth.

be eliminated. In some instances, tumour cells can also become insensitive to many cytotoxic drugs, even those they have not encountered before. This process is called *multidrug resistance* and can be due to an increased ability to pump a range of lipophilic drugs out of the cell (up to 40% of human cancers may develop multidrug resistance). One mechanism for multidrug resistance is when a mutation results in amplification of a gene called *mdr1*. The *mdr1* gene encodes p-glycoprotein, a member of the ABC (ATP binding cassette) transporter superfamily, located on the plasma membrane. It pumps lipophilic compounds, including several key cancer treatment drugs (e.g. the vinca alkaloids and taxanes), out of the cell, decreasing the concentration of the cytotoxic drug inside the cell, thereby reducing its pharmacological effectiveness.

Cytotoxic therapies are not without side-effects for patients. The DNA damage that kills tumorigenic cells may also promote mutations in normal cells, priming further tumour development later in life. The second malignancy is often an acute leukaemia, often two to six years after chemotherapy. The relative risk of developing a second malignancy is low, but still may be around 12-fold compared to individuals with no history of cancer, and depending on which drugs are used. In addition, rapidly dividing normal cells, such as epithelial cells lining the gut or bone marrow cells, will also be targeted by cytotoxic therapies, which may result in extensive damage to these tissues. Stem cell replacement strategies (particularly bone marrow) are currently being developed to replace normal cells that have died as a consequence of the toxicity of conventional cytotoxic therapy.

Table 19.2 Classification of chemotherapeutic drugs.

This is not an exhaustive list, but shows the wide range of molecular targets, most of which you have met earlier in the course.

Class of drug	Examples	Mechanism of action
<i>Alkylating agents</i>	nitrogen mustard, cyclophosphamide	alkylation of DNA bases, which can lead to interstrand DNA cross-linking
<i>Antimetabolites</i>	6-thioguanine, 5-fluorouracil, cytosine arabinoside	base analogues that inhibit nucleic acid synthesis
	methotrexate	folic acid analogue that acts as an antagonist of folate metabolism (implicated in a variety of reactions, such as purine nucleotide synthesis)
<i>Natural products and their derivatives</i> antibiotics	doxorubicin	stabilizes topoisomerase–DNA intermediate (cleavable) complexes, interfering with DNA metabolism
	actinomycin D	RNA synthesis inhibitor
	bleomycin	induces double-strand breaks in DNA
	camptothecin derivatives (from the Chinese <i>Camptotheca</i> tree)	topoisomerase I inhibitor; interferes with DNA metabolism
	vinca alkaloids (derived from the periwinkle plant)	binds to tubulin, disrupting formation of the mitotic spindle
	taxanes (alkaloids from yew trees)	binds to tubulin, but in this case inhibiting disassembly of microtubules
<i>Miscellaneous drugs</i>	cisplatin	intrastrand DNA cross-linking agent; induces DNA repair and cell cycle stalling

Summary of Section 19.2

- 1 Genetic instability accelerates the rate of accumulation of key mutations in cancer-critical genes.
- 2 Genetic instability can be a result of mutation in any of the many genes whose products check, detect and repair damaged DNA.
- 3 The type of genetic instability within a cell reflects the pathway that has been disrupted, e.g. loss of mismatch repair genes leads to a superficially normal karyotype, with many point mutations and short repeats, while loss of ATM or p53 leads to translocations resulting from double-strand DNA breaks.
- 4 The p53 protein normally triggers cell cycle arrest in response to DNA damage, leading to DNA repair or apoptosis if damage is severe. Continued replication in p53-deficient cells leads to increasing genetic damage and chromosomal instability.
- 5 Radiotherapy and chemotherapeutic drugs rely on damaging the DNA of tumour cells so severely that they undergo mitotic failure and die, whilst normal cells, with normal repair mechanisms, either undergo apoptosis or repair the damage and survive.

19.3 Self-sufficiency in growth signals

In Section 19.1, you learnt how mutations in proto-oncogenes, which encode components of growth-inducing signalling pathways, can lead to aberrant overactivity within that pathway. This means that the cell can proliferate independently of the growth factor that normally activates the signalling pathway.

- ☐ Which types of genes coding for components of a typical growth-inducing signalling pathway could be proto-oncogenes? (Refer to Figure 13.3, which outlines the components of these pathways, if you are unsure.)
- ☒ You may have thought of genes that encode the growth factors or their receptors, or of those genes that encode any signalling and/or target proteins in the pathway.

A gene mutation may lead to overexpression of a growth factor or to a hyperactive growth factor. Alternatively, the gene for a growth factor receptor may also become an oncogene following mutation. For example, a mutation in a growth factor receptor gene could lead to expression of a receptor that signals in the absence of the growth factor. Finally, any mutation in genes encoding the intracellular signalling or target proteins (e.g. transcription factors and the cell cycle control proteins) could generate a constitutively activated protein, lead to overexpression of specific protein products and even result in abnormal cell responses (e.g. sustained proliferation).

19.3.1 Gain-of-function mutations in tumour cells

Cellular changes conferring a proliferative or cell survival advantage can be brought about by gain-of-function mutations. Such mutations are extremely common in human tumours, for a number of reasons:

- 1 Only one gene copy need be mutated. In a diploid cell, even if only a proportion of the protein product is constitutively activated, it will still induce the continuous activation of the signalling pathway. In this case, the effect of the mutated gene is described as *dominant*.
- 2 Gain of function can be a result of increased activity or overproduction of a protein, which may occur in a number of ways, as discussed below.
- 3 Some viruses carry versions of eukaryotic genes (viral oncogenes) that are not subject to normal regulation and act within an infected cell as if the cell had acquired a gain-of-function mutation.

An additional explanation for the apparent high rate of gain-of-function mutations detected in human tumours may be because it is relatively easy to identify them by well established assays.

We will now consider how some gain-of-function mutations may occur.

Overproduction of proteins encoded by oncogenes

- Point mutations, insertions or deletions in genes that encode signalling proteins or transcription factors may induce an alteration in their activity, thereby increasing promoter activity and expression of other genes – for example, genes for growth factors or genes required for cell division. A mutation in a promoter region may also increase its activity. Alternatively, a mutation in a gene may stabilize the mRNA transcript of that gene.
- Increased gene copy number ('gene amplification') can occur in a variety of ways, and is a common consequence of increased genetic instability. Vastly amplified areas that are several kilobases long are often detectable as chromosomal changes. The amplified region may be contained within a chromosome (in which case it is called a *homogeneously staining region*), or may be present as separate, small fragments, known as *double minute chromosomes* (see Figure 19.15 in Section 19.3.2).
- Gene rearrangements, ranging from small-scale changes involving a single gene to large-scale alterations visible in the chromosomal structure, may affect gene expression. For example, insertion or acquisition of a powerful promoter upstream of a proto-oncogene can cause increased expression of its protein product. On a larger scale, chromosomal rearrangement may place the proto-oncogene into an actively transcribing area of the genome. Translocation of *myc* in Burkitt's lymphoma is a good example of such a translocation (see Figure 19.16 in Section 19.3.2).

Changes in the activity of proteins encoded by oncogenes

- Point mutations affecting a single amino acid in a regulatory or catalytic part of the protein can change its activity. Some proto-oncogene products such as Ras have several of these potentially critical amino acid sites.
- Deletions or premature truncation can remove a regulatory part of the protein, as we shall see later with Src.
- Chromosome translocations may result in the formation of a novel protein with aberrant activity. For example, in chronic myeloid leukaemia (CML), there is frequently a translocation that results in the fusion of two proteins, creating the hybrid Bcr–Abl fusion protein (see Figure 19.13 in Section 19.3.2).

The mechanisms involved in protein overproduction or activity changes by oncogenes are summarized in Figure 19.6.

The names of the proto-genes and their oncogenic counterparts can create confusion. This reflects the history of the discoveries – often a gene was identified in different systems and by different researchers before it was realized that the same gene was being investigated. Viral versions of cellular proto-oncogenes are called *viral oncogenes* and are given the prefix 'v-', in contrast to cellular proto-oncogenes, which are sometimes given the prefix 'c-' (when discussed in this context). There may also be more than one additional name relating to the cell type and/or the function first attributed to normal and mutant cellular versions of the gene.

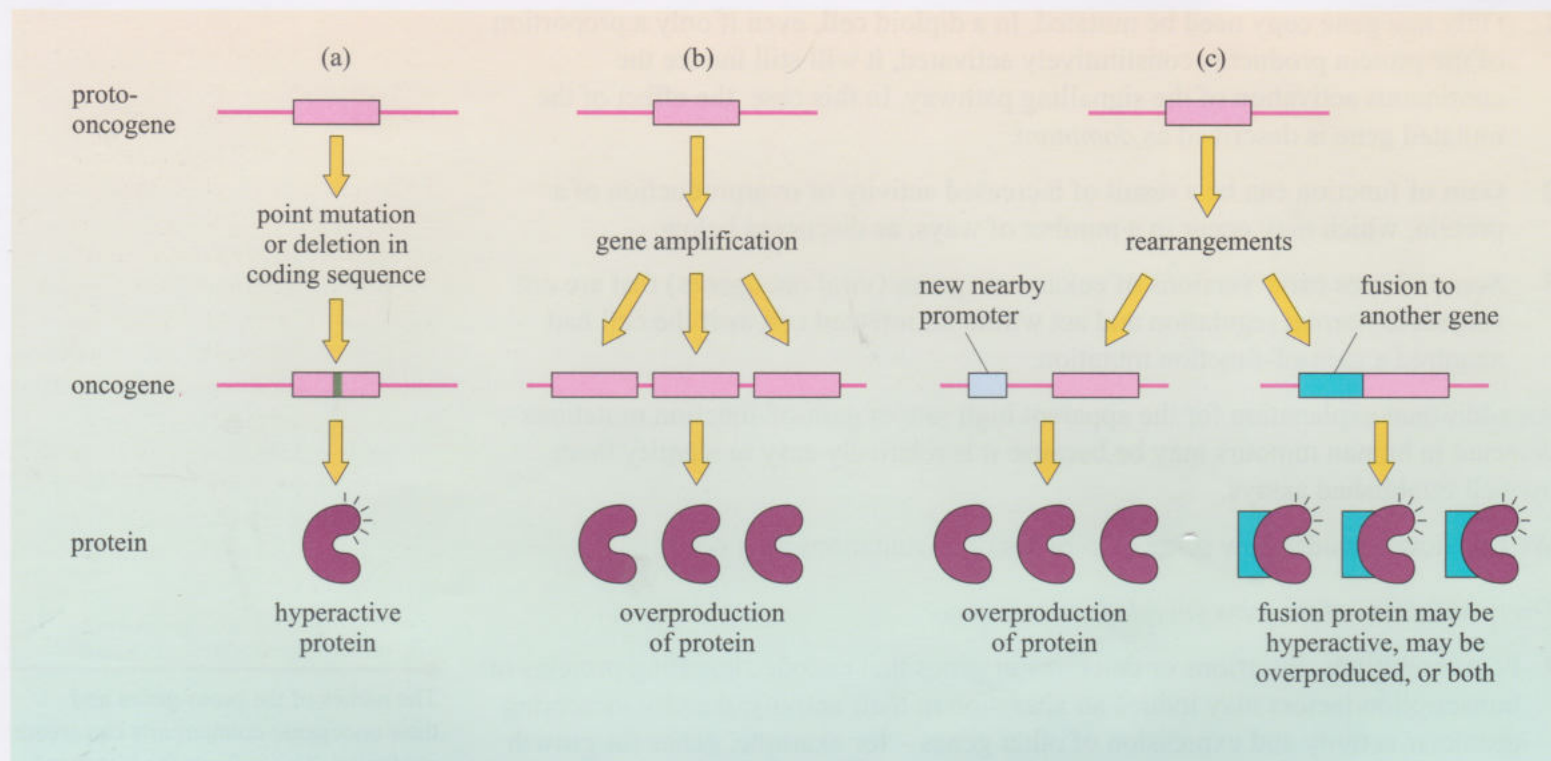


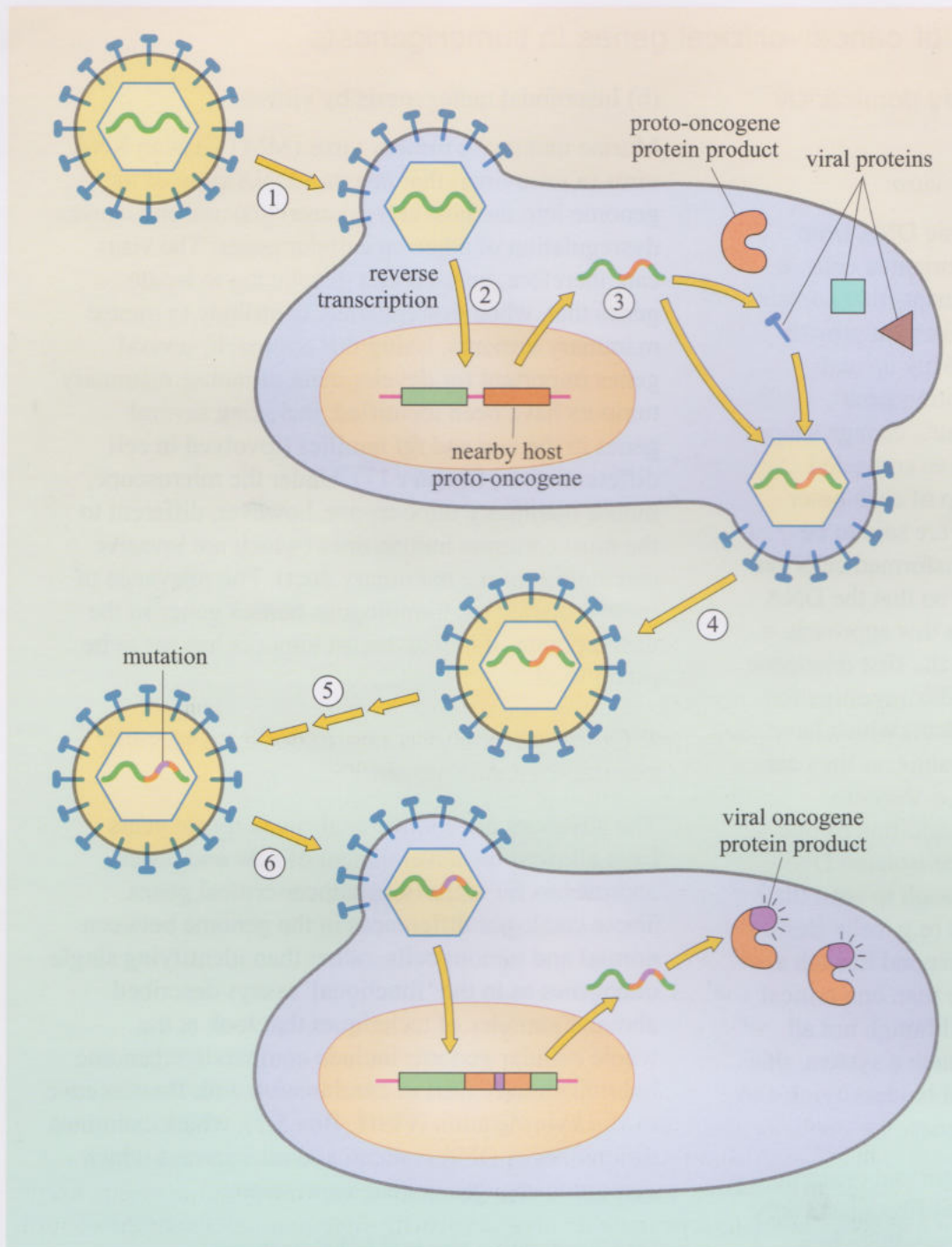
Figure 19.6 A proto-oncogene can become oncogenic by various mechanisms that result either in a hyperactive protein product, or in the increased expression of the protein product, or both. These mechanisms may involve (a) mutations or deletions in either the ORF of a gene or in other DNA regions, (b) gene amplification, or (c) gene and chromosomal rearrangements.

Viral oncogenes

Infection of cells by certain viruses may result either in the overexpression of the host's proteins or in the expression of viral proteins that promote cell growth and survival. Many viruses have at some stage in their evolution acquired copies of their host's genetic material, which may include particular proto-oncogenes. Such genes are called **viral oncogenes** and are likely to have undergone mutations whilst carried by the virus, and these may include gain-of-function mutations that help promote viral replication or survival of the infected cell. Retroviruses, because they insert into the host genome, are particularly likely to have acquired cellular proto-oncogenes (Figure 19.7). A cell that expresses a viral oncogene is susceptible to aberrant control of cell growth. It is also possible that the viral genome inserts itself so that genes in the host genome involved in growth signalling fall under the control of an overactive viral promoter, e.g. murine mammary tumour virus (MMTV; see Box 19.2).

It is worth noting that although viral oncogenes or upregulation of cellular genes by viral promoters may be of some benefit to the virus, it is often at the heavy cost of disruption to the normal viral genome, so an oncogenic virus tends to exist as a subpopulation, requiring co-infection of host cells with normal versions of the virus too. A mutant version of *ras* was the first viral oncogene to be identified (see Section 19.3.2), and many other cellular proto-oncogenes, including *src*, were first identified by the discovery of their viral counterparts. Note that retroviruses that carry oncogenes have not been found in humans, most examples coming from avian and murine sarcomas and leukaemias.

Some human cancer-associated viruses do, however, cause cell proliferation and tumour development in other ways. One human retrovirus that is known to induce

**Figure 19.7**

Acquisition of viral oncogenes by retroviruses. (1) Infection by retroviruses involves fusion of the viral envelope with the plasma membrane of the host cell and (2) unpacking and reverse transcription of retroviral RNA followed by insertion of viral DNA into the host DNA. (3) Viral replication involves the transcription of viral RNA and expression of viral proteins that mediate the production of mature infectious viruses. Note that a proto-oncogene situated nearby in the host DNA can accidentally be picked up by the viral RNA at the time of transcription (depicted in orange within the green viral RNA) and its protein product can be translated together with the other viral proteins. (4) The newly formed virus carries the host proto-oncogene within its genome. (5) Successive rounds of viral infection and replication may lead to the mutation of the proto-oncogene (depicted in purple within the orange and green viral RNA), generating a viral oncogene. (6) Viral infection and replication in a new host cell lead to overproduction (as the viral oncogene is under the control of a viral promoter) and/or expression of a hyperactive form of the protein product encoded by the viral oncogene.

tumorigenesis directly is HTLV-1 (human adt T cell leukaemia virus). An HTLV-1 gene, *tax*, is a transcriptional activator for both viral and cellular genes. Several other viruses, including Epstein-Barr virus (EBV) and papillomaviruses, will be discussed later in this chapter.

S320, Book 3, Section 4.1 describes several virally induced tumours in humans.

As mentioned above, gain-of-function mutations are relatively easy to identify by well established assays. Box 19.2, Section 1 describes the classical ways of identifying oncogenes. These classical techniques enabled the identification of many dominant, gain-of-function mutations. Box 19.2, Section 2 describes currently used high-throughput methods based on genomic analysis.

Box 19.2 Identification of cancer-critical genes in tumorigenesis

1 Functional assays to identify dominantly acting oncogenes

(a) Transformation of fibroblasts *in vitro*:

By introducing fragments of genomic DNA from tumour cells into cultured non-tumorigenic cells, it is possible to isolate the DNA fragments that contain mutated genes responsible for the aberrant growth in the tumour. In this assay, cells taking up and expressing a mutant, growth-promoting gene proliferate more than their neighbours, change shape and continue to divide after they have contacted other cells, so that cells grow on top of each other in a disorderly fashion. These cells are said to be *transformed* (see Figure 19.8). Transformed cells can then be isolated, and grown further so that the DNA can be extracted for analysis. Using this approach, a mutant version of the *ras* gene was the first oncogene to be identified. The cells used for this investigation are fibroblast cell lines (usually rodent) which have already undergone some genetic change, as they can proliferate indefinitely in culture (i.e. they are *immortalized*; Section 18.3.3). The addition of one more aberrant gene (contained in the isolated DNA fragments from tumour cells) is enough to send their growth out of control. Normal cells (e.g. cells freshly isolated from a tissue) would not respond in such a way, because it would require more than one critical genetic lesion to transform them. Although not all oncogenes would be detectable in such a system, this approach has been extremely useful in identifying and characterizing many human oncogenes.

Further assays can be used to confirm and examine the extent of transformation. Normal fibroblasts only grow in culture if they are allowed to adhere to a suitable supporting surface, such as glass or plastic. Transformed fibroblasts, however, display anchorage-independent growth, which means that they do not require a surface to attach to in order to grow. Such cells are isolated for their ability to grow as a colony in a semi-solid medium such as agar, which prevents adherence to the culture dish surface (Figure 19.8b). Finally, transformed fibroblasts can form tumours in immunologically deficient mice, whereas normal cells do not. (Note that in immunologically normal mice, transplanted cells are usually treated as a foreign tissue graft and are destroyed.)

(b) Insertional mutagenesis by viruses:

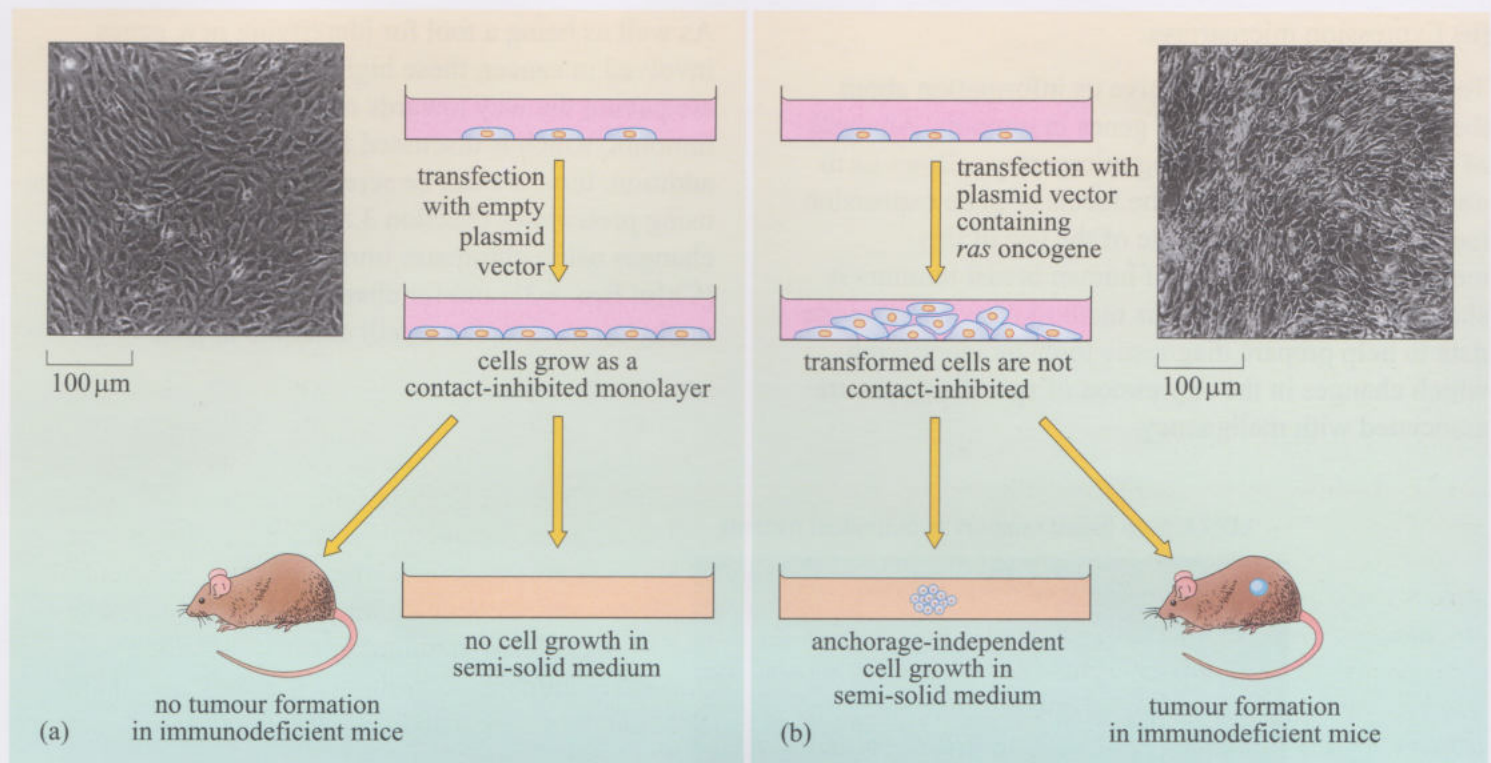
Murine mammary tumour virus (MMTV) is an RNA virus (a retrovirus) that inserts a DNA copy of its genome into the host cell (mouse) genome and causes dysregulation of adjacent cellular genes. The virus can, therefore, be used as a genetic tag to locate genes that, when dysregulated, contribute to mouse mammary tumours. Using this approach, several genes important for development of mouse mammary tumours have been identified, including several genes in the *wnt* and *fgf* families (involved in cell differentiation, Chapter 17). Under the microscope, mouse mammary tumours are, however, different to the most common human ones (which are invasive carcinomas of the mammary duct). The relevance of these mutations in homologous human genes in the development of human breast tumours has yet to be established.

2 Genomic analysis techniques to identify cancer-critical genes

The advances in genomic analysis in recent years have allowed the development of new and faster approaches for identifying cancer-critical genes. These catalogue differences in the genome between normal and tumour cells, rather than identifying single oncogenes as in the 'functional' assays described above. Examples of techniques that look at the whole cellular genome include comparative genome hybridization (CGH) in combination with fluorescence *in situ* hybridization (FISH; Box 5.1), which examines differences in DNA content, and microarrays which determine changes in gene expression.

(a) Comparative genome hybridization:

Comparative genome hybridization (CGH) compares the differences in DNA content between tumour and normal cells. FISH can then be used to identify cancer-critical genes and to compare their copy number on normal metaphase chromosomes. The principle of CGH is shown in Figure 19.9. The resolution by which differences between two chromosomes can be detected by FISH is, however, poor (around 10 Mb). Applying microarray technology to CGH and FISH improves this resolution, and this approach can be used to identify regions of the genome that are deleted or present in multiple copies, down to an accuracy of 100 kb.



▲ Figure 19.8 Fibroblast cell lines can be transformed by oncogenes. (a) The appearance of a non-tumorigenic fibroblast cell line in tissue culture is shown in the phase contrast micrograph. (b) The abnormal morphology and growth characteristics of an immortalized fibroblast cell line transformed by addition of oncogenic *ras*. Proliferation of transformed cells is anchorage-independent, and they can induce tumours in immunodeficient mice.

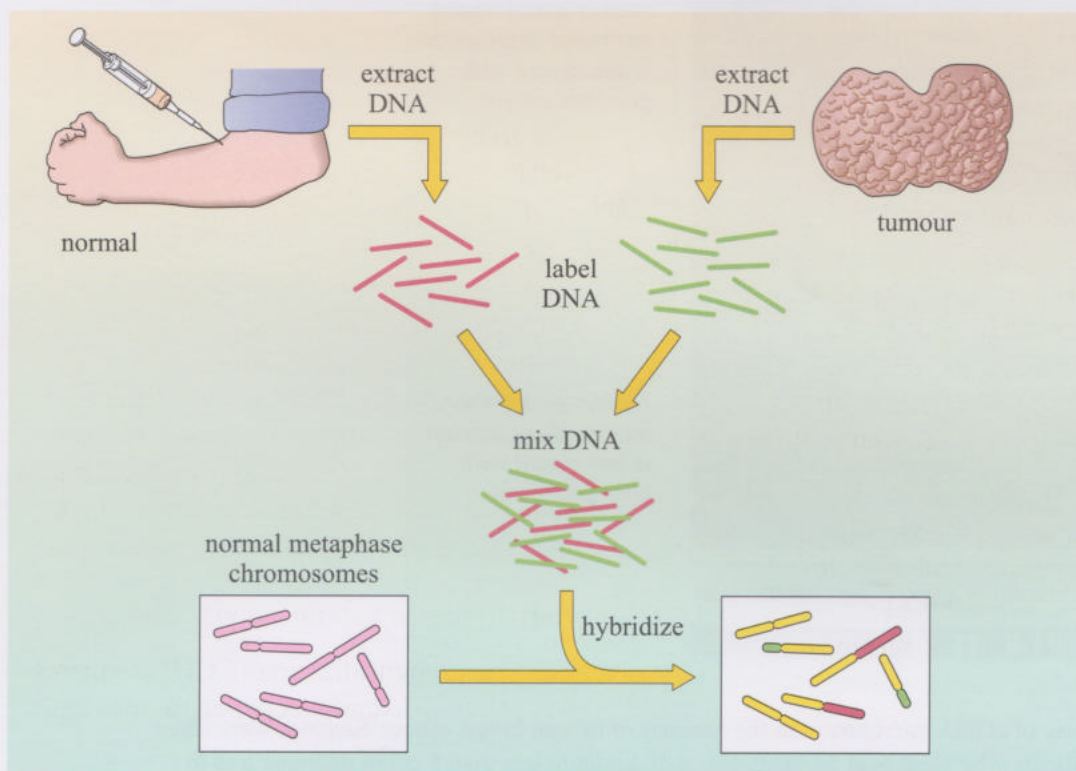


Figure 19.9 Comparative genome hybridization. DNA is extracted from both tumour and reference (normal tissue) samples and labelled using different-coloured fluorescent dyes. In this case, tumour DNA is labelled with a green fluorophore, whereas reference DNA is labelled with a red fluorophore. The labelled DNA samples are mixed together under specific conditions. This mixture of DNA is first denatured and then competitively hybridized onto denatured normal metaphase chromosomes. Complementary strands of normal and tumour DNA anneal to chromosomal target DNA, forming double-stranded DNA (tumour and normal DNA that anneal to each other are subsequently washed away). The images are captured digitally and analysed using computer software to obtain a graphical read-out of changes in DNA copy number. In the diagram, regions that appear red have a decrease in DNA copy number in the tumour compared to normal, whereas those that are green have an increased DNA copy number compared to normal. The yellow areas represent segments of the chromosome in which the DNA copy numbers are the same between the reference and tumour samples.

(b) Expression microarrays:

Techniques such as CGH give us information about the presence or absence of genes in tumours. Analysis of tumour cell mRNA using microarrays allows us to obtain information about the levels of gene expression (see Box 10.4). An example of the use of this technique in the analysis of human breast tumours is shown in Figure 19.10. This methodology can provide data to help prepare diagnostic tools by identifying which changes in the expression of specific genes are associated with malignancy.

As well as being a tool for identifying new genes involved in cancer, these high-throughput techniques are paving the way towards molecular profiling of tumours, which is discussed further in Box 19.4. In addition, tumours can be screened for protein changes using proteomics (Section 3.8.3), for chromatin changes using chromatin immunoprecipitation (ChIp; Box 5.3), and for changes in genomic DNA methylation, a topic we will examine in Box 19.5.

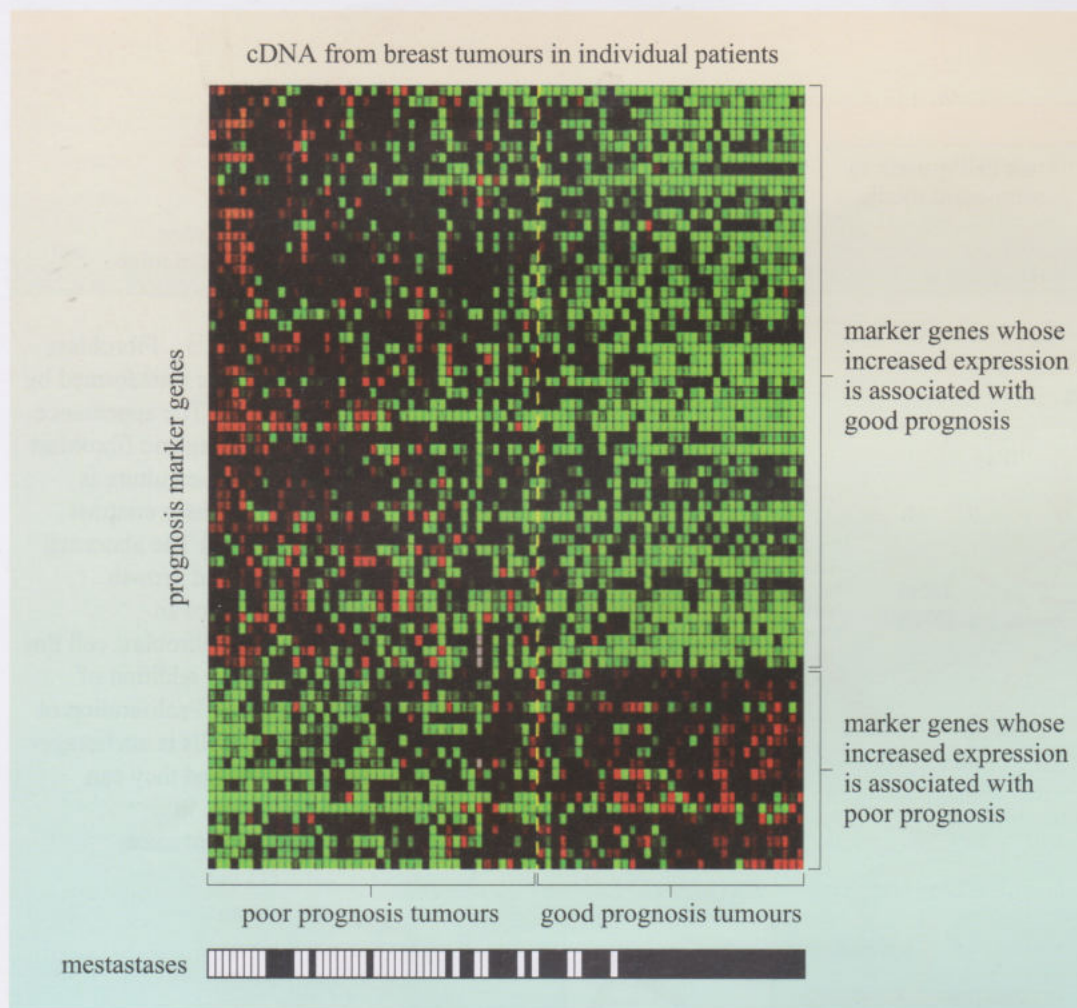


Figure 19.10 An example of the use of cDNA microarrays in the analysis of human breast cancer. Samples were taken from 78 sporadic breast tumours of patients who were over 55 years old, with tumours less than 5 cm in diameter and in whom the tumour had not metastasized to the local lymph nodes (individual tumour samples are arranged in columns from left to right in the array). cDNA from each patient (made from tumour mRNA) was hybridized on a microarray containing a panel of 70 DNA marker genes, chosen because their expression is known to correlate with the severity of tumours (individual prognosis marker genes are arranged in rows from top to bottom in the array). In this experiment, increased gene expression in tumours compared to controls is shown in green, whereas the reverse is shown in red (no change in gene expression is shown in black). The genes are positioned according to whether their expression correlates with a good prognosis (upper part of array) or not (lower part). Patients with tumours to the right of the dashed line have a pattern of gene expression associated with a better prognosis, whilst patients to the left have a worse prognosis. The bar at the bottom shows in black those patients who remained disease-free after five years. (Data from van't Veer *et al.*, 2001)

19.3.2 Many components of growth factor pathways are proto-oncogenes

In Chapter 13, we described the major signalling pathways involved in cellular responses to an extracellular signal such as a growth factor. Any gene that encodes a component along these signalling pathways, from the extracellular growth factor or hormone through to the receptor (cell surface or intracellular), intracellular signalling proteins (e.g. protein kinases, G proteins), and target proteins (e.g. transcription factors) is potentially a proto-oncogene. What these genes have in common is that mutations resulting in hyperactive proteins or increased gene expression lead to enhanced signalling capacity. Table 19.3 illustrates the variety of signalling molecules whose genes have been found to be proto-oncogenes. We will now look at key examples of the involvement of each type of signalling molecule in the development of certain tumours.

Table 19.3 Oncogenes involved in growth-inducing signalling pathways.

Oncogene/proto-oncogene	Normal role of protein product
<i>v-sis</i>	platelet-derived growth factor
<i>erbB-1</i>	epidermal growth factor receptor
<i>erbB-2/neu/HER2</i>	heregulin receptor
<i>fms</i>	CSF-1 receptor
<i>kit</i>	stem cell factor receptor
<i>trkA</i>	nerve growth factor receptor
<i>erbA</i>	thyroid hormone receptor (nuclear)
<i>H-, K- and N-ras</i>	small GTPases
<i>bcr-abl</i> fusion gene	tyrosine kinase (not found in normal cells)
<i>lck</i>	tyrosine kinase, activation of T lymphocytes
<i>src</i>	tyrosine kinase, signalling activated by growth factors
<i>raf</i>	serine-threonine kinase, activates MEK
<i>fos</i>	transcription factor
<i>jun</i>	transcription factor
<i>myc</i>	transcription factor

Growth factors

One of the best-known examples of a growth factor proto-oncogene is the gene encoding platelet-derived growth factor (PDGF). This oncogene was first identified when a section of the gene encoding one of the PDGF chains was discovered in a simian sarcoma virus isolated from a fibrosarcoma (a type of sarcoma originating from connective tissue fibroblasts) in a woolly monkey.

Recall from Chapter 13 that PDGF is a dimer itself and activates its receptor by binding to two PDGF receptor chains which then dimerize and cross-phosphorylate each other. The related viral oncogene, called *v-sis*, was found to be able to transform fibroblasts. It is thought that the virally encoded protein is a truncated homologue of PDGF that acts in an autocrine manner to stimulate proliferation in infected cells that express PDGF receptors and hence induce transformation and tumorigenesis.

PDGF has been shown to be important not only in the growth of virus-induced transformed cells but also in some human brain tumours derived from glial cells (called gliomas). Moreover, it has been postulated that autocrine stimulation by growth factors can help explain how metastatic cells can survive in alien tissues, and create secondary tumours (see Section 19.8).

- ☐ Why might you expect cells that are incorrectly located within the body, not to survive?
- ☒ Many cells require adhesion molecule-dependent signals to survive (Section 16.1.1) and displaced cells would not interact with the appropriate neighbouring cells to receive these signals.

Growth factor receptors

Many human tumours show gene amplification and/or overexpression of receptor tyrosine kinases (RTKs) or tyrosine kinase-associated receptors, which results in an increase in the response of tumour cells to their growth factor ligand. Moreover, as indicated above, some tumour cells overexpress and secrete the growth factors that can act on their own growth factor receptors by autocrine action.

- ☐ What is the general mechanism of activation of RTKs?
- ☒ Growth factor binding typically induces receptor dimerization, causing the intracellular kinase domains to cross-phosphorylate each other (autophosphorylation) on tyrosine residues that become docking sites for intracellular PTB- and SH2-containing proteins (See Figures 13.24 and 13.31).

Mutations in cancer-critical genes can cause other human diseases. For example, some mutations in the gene for FGFR3 produce developmental skeletal disorders.

The heregulin receptor belongs to the EGF receptor family of RTKs (Figure 13.23b), and is encoded by the *HER2* gene (for human EGF receptor related gene, also called *erbB-2*, and also called *neu* because of its identification in neuroblastomas, i.e. tumours derived from nervous tissue). The HER2 receptor is involved in mammary epithelium development, and gene amplification and overexpression of this receptor is seen in about 25% of human breast cancers. Box 19.3 describes therapies that are being developed to interfere with HER2 receptor function.

Mutations may also result in the constitutive activation of RTKs, which are otherwise expressed at normal levels. Constitutive activation of RTKs may be either by inducing dimerization or by continuous activation of the enzymatic domain. A good example of RTK activity changes in tumours is provided by a receptor of the fibroblast growth factor family called FGFR3, which shows different mutant forms in different types of human tumour. In some human carcinomas, a change in the extracellular domain creates a new cysteine residue that forms a disulfide bridge with another mutant FGFR3 receptor, resulting in their dimerization and activation. In many human multiple myelomas, a mutation leads to an amino acid change in the activation loop of the kinase domain, which renders FGFR3 constitutively active.

Box 19.3 Therapies that target receptors in human cancer

Intracellular hormone receptors: tamoxifen

Our understanding of the oestrogen requirements of mammary epithelium has led to the development of antagonists for the treatment of some human breast cancers. These antagonists bind competitively to oestrogen receptors, thereby inhibiting the oestrogen-mediated proliferation of mammary epithelium and of associated tumours that retain some oestrogen dependency. The oestrogen antagonist *tamoxifen* (see Figure 19.11) is used to restrict the growth of hormone-responsive metastatic breast tumours. Oestrogen antagonists have the additional advantage of less severe side-effects than cytotoxic drugs, as they are targeted to cells that express the oestrogen receptor. However, acquired resistance of tumour cells to the drug is still a major problem.

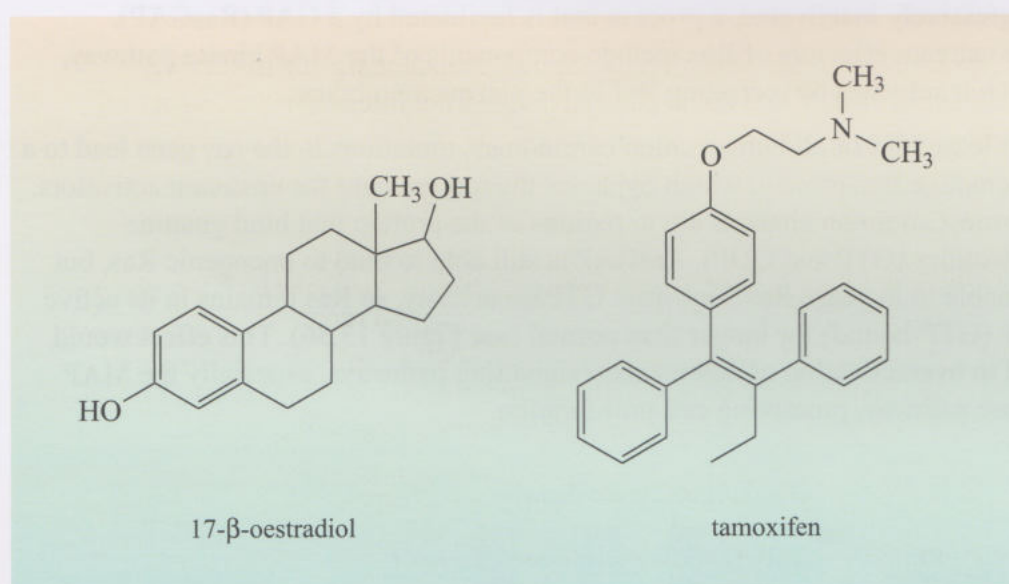


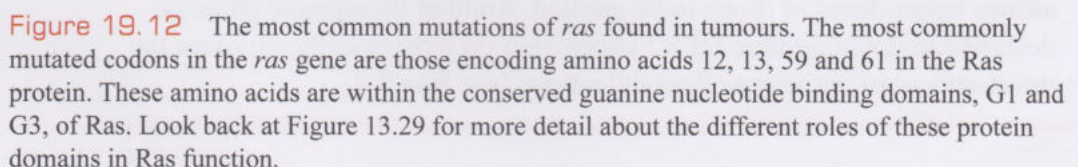
Figure 19.11 Molecular structures of 17-β-oestradiol (an oestrogen) and the oestrogen antagonist tamoxifen.

Cell surface growth factor receptors: HER2 antibodies and antisense strategies

Monoclonal antibodies can be used to block tumour cell surface receptors. An example of this strategy is Herceptin, a monoclonal antibody that blocks the activity of the heregulin receptor encoded by *HER2*. Herceptin has been used to treat breast tumours that overexpress *HER2*. *HER2* is also the target of another monoclonal antibody therapy but, in this case, the antibody not only binds to *HER2* on the tumour cell but also induces cell lysis. This is achieved by attaching cytotoxic drugs (or enzymes that locally cleave and activate prodrugs injected intravenously) to the monoclonal antibody specific for *HER2*, thereby inducing localized toxic effects on the tumour cell that the antibody binds to. This method avoids general toxicity to normal cells and allows larger doses of drugs to be applied. Another therapeutic strategy, designed to downregulate *HER2* expression on tumour cells, involves the development of antisense oligonucleotides (see Box 5.2).

Ras

In at least 50% of all human colon carcinomas, mutations in the *ras* gene lead to a hyperactive Ras protein, which bypasses the requirement for upstream activators. The most common changes are in regions of the protein that bind guanine nucleotides (GTP and GDP). RasGAP is still able to bind to oncogenic Ras, but is unable to increase Ras's intrinsic GTPase activity, so Ras remains in its active state (GTP-bound) for longer than normal (see Figure 13.36). This effect would lead to overactivation of downstream signalling pathways, especially the MAP kinase pathway, promoting cell proliferation.



- ☐ Can you think of a mutation of another gene that would have similar consequences to that leading to hyperactive Ras?
- ☒ A mutation leading to loss of RasGAP expression or to a decrease in its activity. In these cases, the gene encoding RasGAP is a tumour suppressor gene.

Src

The *src* gene was first identified as a proto-oncogene in a transforming retrovirus, in this case Rous sarcoma virus. You have encountered the Src protein before, exploring its domains in Chapter 3 and its role in signalling pathways in Chapter 13.

- ☐ How is the activity of the Src protein regulated?
- ☒ Src consists of a kinase domain, an SH2 domain, an SH3 domain and a C-terminal inhibitory region, which is normally phosphorylated on Tyr 527. The inhibitory region binds to the SH2 domain, keeping the protein in a compact, inactive conformation. This inhibitory binding is relieved when the SH2 domain binds instead to a phosphotyrosine residue on a different signalling protein and Src is activated.

Cells infected with the Rous sarcoma virus express, in addition to normal Src (c-Src), a mutant viral form of Src (v-Src), which is constitutively active and usually leads to aberrant proliferation of infected cells. v-Src is characterized by the substitution of the 19 C-terminal amino acids (from residue 514 to 533) in c-Src by a sequence of 12 unrelated amino acids.

- ☐ Why does the mutation of the *src* gene leading to substitution in the C-terminus of the protein cause the tyrosine kinase activity of v-Src to be constitutively active?
- ☒ v-Src is constitutively active because there is no tyrosine in position 527 and therefore there is no inhibitory action by the C-terminal region. Tyr 416 then becomes phosphorylated and activates the kinase domain (see Section 3.4.3).

Abl

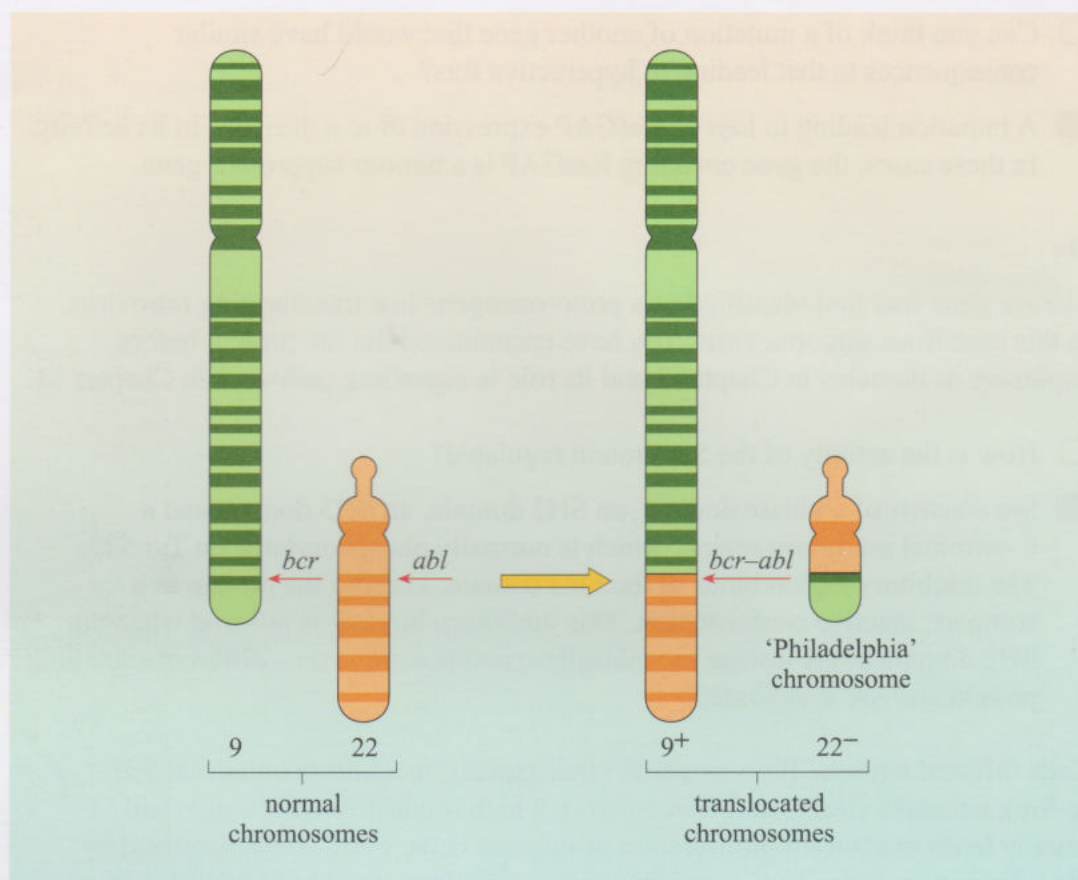
The *abl* proto-oncogene encodes a protein tyrosine kinase that was first identified in mutated form in a retrovirus (Abelson murine leukaemia virus). The viral gene is expressed as a fusion protein (v-Abl) in which a portion of the viral capsid protein Gag, replaces the N-terminal SH3 domain of c-Abl. Removal of the SH3 domain constitutively activates the tyrosine kinase and the Gag moiety is modified by myristoylation, which targets the fusion protein to the cytosolic leaflet of the plasma membrane; both modifications are important in the transforming activity of v-Abl.

Another mutant oncogenic form of *abl* is involved in over 95% of human chronic myelogenous leukaemia (CML) cases, a condition that arises from B cell precursors. In CML, there is a chromosomal translocation whereby the end of the large chromosome 9 (which includes a segment of the *abl* gene) is exchanged with the end of the small chromosome 22. This results in a characteristically small chromosome 22 (called the Philadelphia chromosome after the city where it was discovered) and a chromosome 9 that carries most of the *abl* gene relocated downstream of *bcr* (breakpoint cluster region; see Figure 19.13). Although the

The American researcher Peyton Rous shared a Nobel Prize in 1966 for his life's work on identifying tumour-inducing viruses. These viruses were mostly isolated by scouring the local market for sick-looking chickens, which were often found to have sarcomas and leukaemias. The first virus he identified in 1910 was found to contain the *src* gene 70 years later. Src was the first ever protein tyrosine kinase to be isolated.

Figure 19.13

In chronic myelogenous leukaemia, translocation between chromosomes 9 and 22 (red arrows) can result in the juxtaposition of *bcr* and *abl* (from chromosome 22) to create a *bcr-abl* oncogene on chromosome 9 (and a characteristically small 'Philadelphia' chromosome 22).



breakpoint can be anywhere within a 50 kb region, similar fusion proteins are generated in different CML cases due to the particular arrangements of introns/exons belonging to the *abl* and *bcr* genes. The resultant fusion protein, Bcr-Abl, contains the kinase domain of Abl which is constitutively active because, as in the v-Abl fusion protein, the normal N-terminal SH3 domain of Abl has been replaced by an unrelated peptide. The creation of such a fusion protein, which is never detected in normal cells but is consistently generated in certain tumour types, has made possible special therapeutic opportunities as described in Box 19.4.

Box 19.4 Tyrosine kinase activity inhibitors in human cancer

Human chronic myelogenous leukaemia is characterized by the synthesis of an oncogenic Bcr-Abl fusion protein. A drug has been identified, named Gleevec, that specifically binds to the kinase domain of Abl in the Bcr-Abl fusion protein and blocks binding of ATP, thereby inhibiting its tyrosine kinase activity (Figure 19.14).

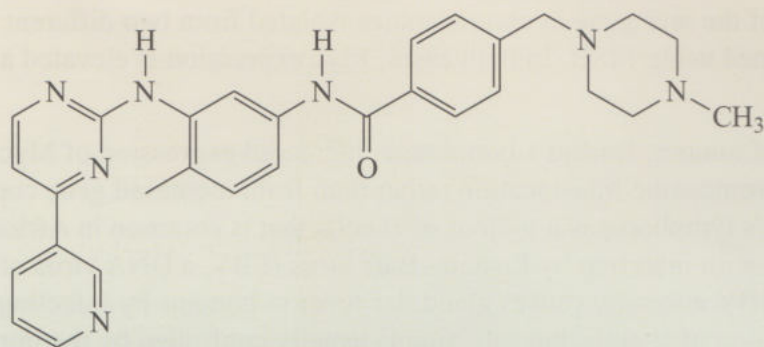
Other drugs with broad-range specificity have been developed to inhibit the tyrosine kinase activity of RTKs of the EGF receptor family (including HER2) that are overexpressed in many human tumours, including colorectal, pancreatic and non-small cell lung cancer. Like Gleevec, they bind, and block the binding of ATP, to the kinase domain of EGF receptors. These strategies have been used in

combination with others targeted to specific receptors (e.g. Herceptin) for the treatment of the most aggressive tumours that show concomitant expression of the EGF receptor and HER2.

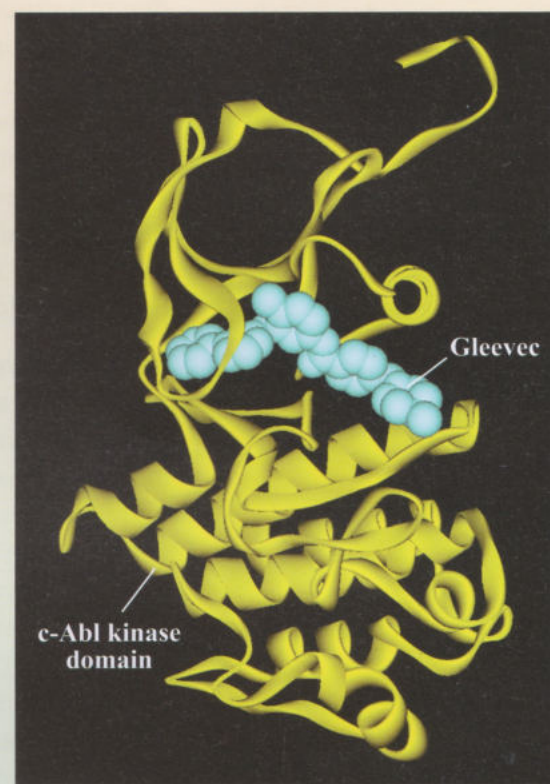
At the time of writing (summer 2004), the advent of therapies tailored for individual cancer patients has moved a step closer to reality with the publication of a study that explains why tumours of some lung cancer patients regress after treatment with a tyrosine kinase inhibitor, Gefinitib, while those of other patients do not. This study showed that sensitivity to Gefinitib was associated with specific mutations in the activation loop of the EGF receptor that are only present in a proportion of lung tumours, whereas Gefinitib-resistant tumours showed mutations that

affected other domains of the EGF receptor. This discovery will facilitate the choice of therapy by clinicians treating lung cancer patients, as they will be

able to profile the molecular lesions of each tumour and identify in advance those patients who will benefit from Gefinitib therapy.

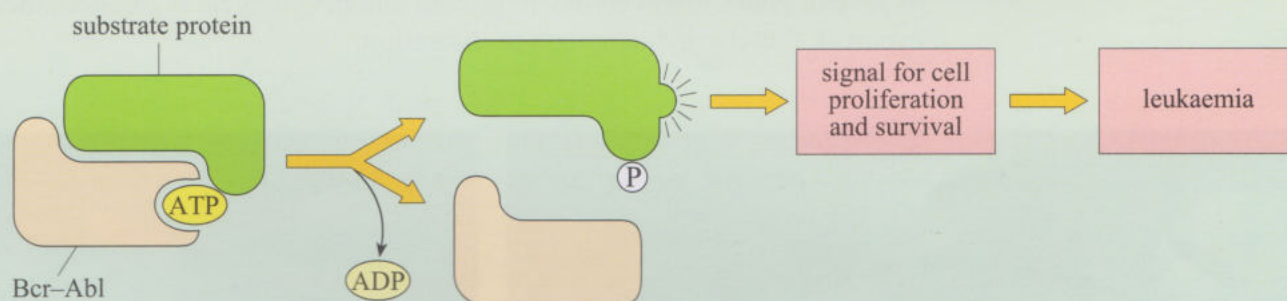


(a)

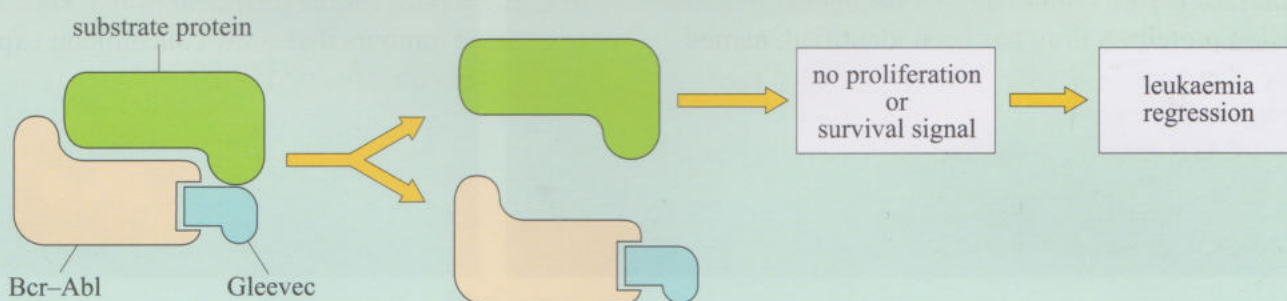


(b)

(i) Bcr-Abl active



(ii) Bcr-Abl blocked with Gleevec



(c)

Figure 19.14 Gleevec blocks the activity of Bcr-Abl in chronic myelogenous leukaemia. (a) The structure of Gleevec. (b) Gleevec (space-filling in blue) occupying the ATP binding pocket of the kinase domain of c-Abl (ribbon structure). (c) Schematic diagram showing how Gleevec prevents ATP from occupying its normal site in Bcr-Abl, thus preventing Bcr-Abl from using the terminal phosphate of ATP to phosphorylate target signalling proteins.

Targets of signal transduction pathways

Many sequence-specific transcription factors and cell cycle control proteins are also dysregulated in cancer. The proto-oncogene *myc* is frequently involved in human tumours. The *myc* gene itself is often found in multiple copies within cells due to a process known as DNA amplification. The exact mechanism by which this process occurs is unclear but DNA amplification can lead to tens or hundreds of copies of the gene involved. As mentioned earlier, sites of amplification can occur within the chromosome (homogeneously staining regions) or, in other cases, the multiple copies of the gene are found on new small chromosomes (double minute chromosomes) (see Section 19.3.1). Figure 19.15 shows the different location of multiple copies of the *myc* gene in chromosomes isolated from two different tumours and stained using FISH. In both cases, *Myc* expression is elevated above normal levels.

In another type of tumour, Burkitt's lymphoma, increased expression of *Myc* results from a chromosome translocation rather than from increased gene copy numbers. Burkitt's lymphoma is a tumour of B cells that is common in Africa, and is associated with infection by Epstein–Barr virus (EBV, a DNA virus of the herpes family). EBV normally causes glandular fever in humans by infecting and causing proliferation of B cells, but infection is usually controlled by the immune system. However, in a small fraction of cases, there is a malignant transformation of B cells, causing Burkitt's lymphoma. In these cases, a chromosomal translocation has occurred (Figure 19.16), which results in the *myc* gene being translocated from chromosome 8 to chromosome 14. Translocation of *myc* brings it under transcriptional regulation by the promoters of genes coding for antibodies. Since antibodies are produced in large quantities by activated B cells, the result is overproduction of *myc* mRNA and the *Myc* protein. *Myc* promotes transcription of several genes involved in cell growth and in cell cycle regulation, including cyclin D, thereby inducing cell proliferation.

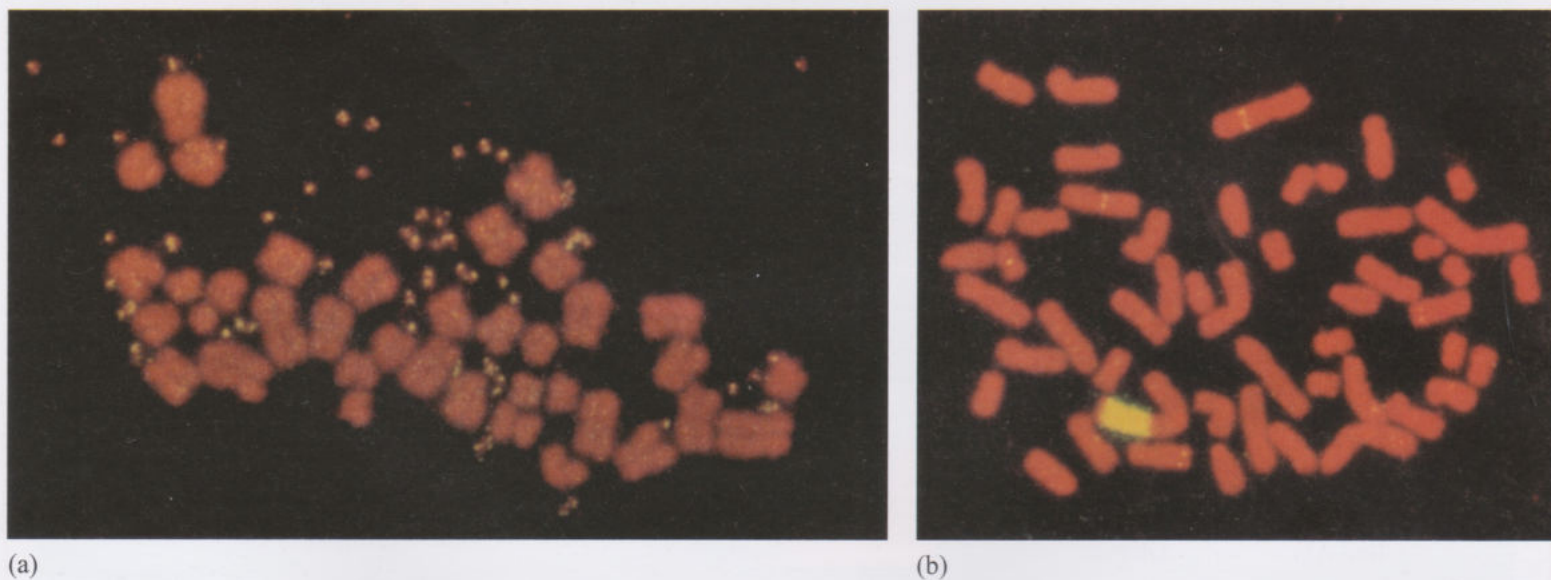
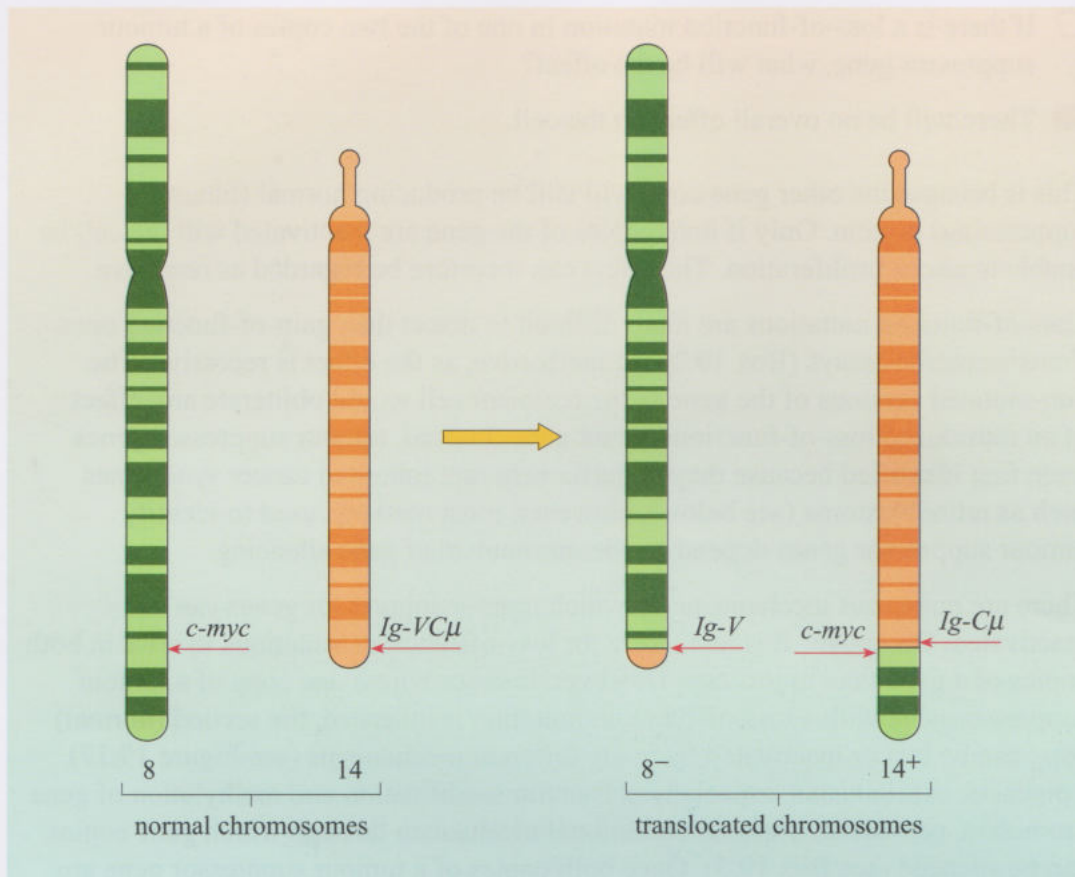


Figure 19.15 Gene amplification in tumour cells. In both micrographs, the chromosomes isolated from two different tumours are stained with a red fluorescent dye, and the aberrantly large number of copies of the *myc* gene have been detected by FISH using a green fluorescent DNA probe (appearing as yellow due to overlapping green and red staining). (a) Double minute chromosomes; here the *myc* gene copies are seen as many paired yellow specks, separate from the larger, normal chromosomes. (b) Homogeneously staining region; here the multiple *myc* gene copies are all contained within one region of one of the cell's chromosomes.

**Figure 19.16**

Burkitt's lymphoma with tumorigenic growth of B cells is characterized by a translocation (red arrows) that moves the *myc* gene from chromosome 8 to chromosome 14. Chromosome 14 contains gene segments encoding the heavy chains of antibodies (*Ig-V Cμ*). In B cells with this translocation, *myc* falls under the control of a promoter that regulates the expression of antibody genes, and it is therefore inappropriately expressed.

- ☐ How do elevated cyclin D levels regulate entry into S phase of the cell cycle?
- ☒ Cyclin D is required to allow the cell to pass through the restriction point (a G1 checkpoint). Overproduction of cyclin D would therefore allow the cell to progress past this G1 checkpoint and into S phase (see Section 8.3.4).

19.4 Insensitivity to growth-inhibitory signals

As well as positive signals to proliferate, cells have many checkpoints and pathways that limit growth and division. The signal can be either externally derived, i.e. a growth-inhibitory factor, or a result of checking the internal state of the cell. Several of the key tumour suppressor proteins operate as growth-inhibitory signals. We will look at two well-studied examples in this section, Rb and p53. First, though, we will examine the ways in which tumour suppressor genes can become inactivated by mutation.

19.4.1 Loss-of-function mutations in tumour cells

Tumour suppressor genes encode proteins that normally limit cellular proliferation, i.e. under normal circumstances they suppress tumour formation. If a tumour suppressor gene sustains a mutation that leads to a loss of function, then cell division can proceed unchecked. This is the opposite of the situation when a proto-oncogene is mutated to become oncogenic, which in this case is a gain-of-function mutation.

- ☐ If there is a loss-of-function mutation in one of the two copies of a tumour suppressor gene, what will be the effect?
- ☒ There will be no overall effect on the cell.

This is because the other gene copy will still be producing normal (tumour suppressing) protein. Only if *both* copies of the gene are inactivated will the cell be unable to check proliferation. The effect can therefore be regarded as *recessive*.

Loss-of-function mutations are more difficult to detect than gain-of-function ones. Transformation assays (Box 19.2) are ineffective, as the effect is recessive. The non-mutated versions of the gene in the recipient cell would obliterate any effect of an introduced loss-of-function mutant gene. Instead, tumour suppressor genes were first identified because they underlie very rare inherited cancer syndromes such as retinoblastoma (see below). However, most methods used to identify tumour suppressor genes depend on the mechanism of gene silencing.

There are numerous mechanisms by which tumour suppressor genes can be inactivated. Obviously, it is less likely for loss-of-function mutations to arise in both copies of a gene than in just one. However, in cases where one copy of a tumour suppressor gene with a loss-of-function mutation is inherited, the second (normal) copy can be lost or inactivated by many different mechanisms (see Figure 19.17). Epigenetic mechanisms, especially chromatin modification and methylation of gene promoters, provide an important additional mechanism through which gene copies can be silenced (see Box 19.5). Once both copies of a tumour suppressor gene are lost, the tumorigenic potential of the cell may be even greater than that of cells sustaining a single gain-of-function mutation in an oncogene. This is because a cell may be able to progress through cell cycle checkpoints even though it has other genetic lesions (as discussed in Section 19.2). This is a major mechanism of genetic instability in tumorigenesis.

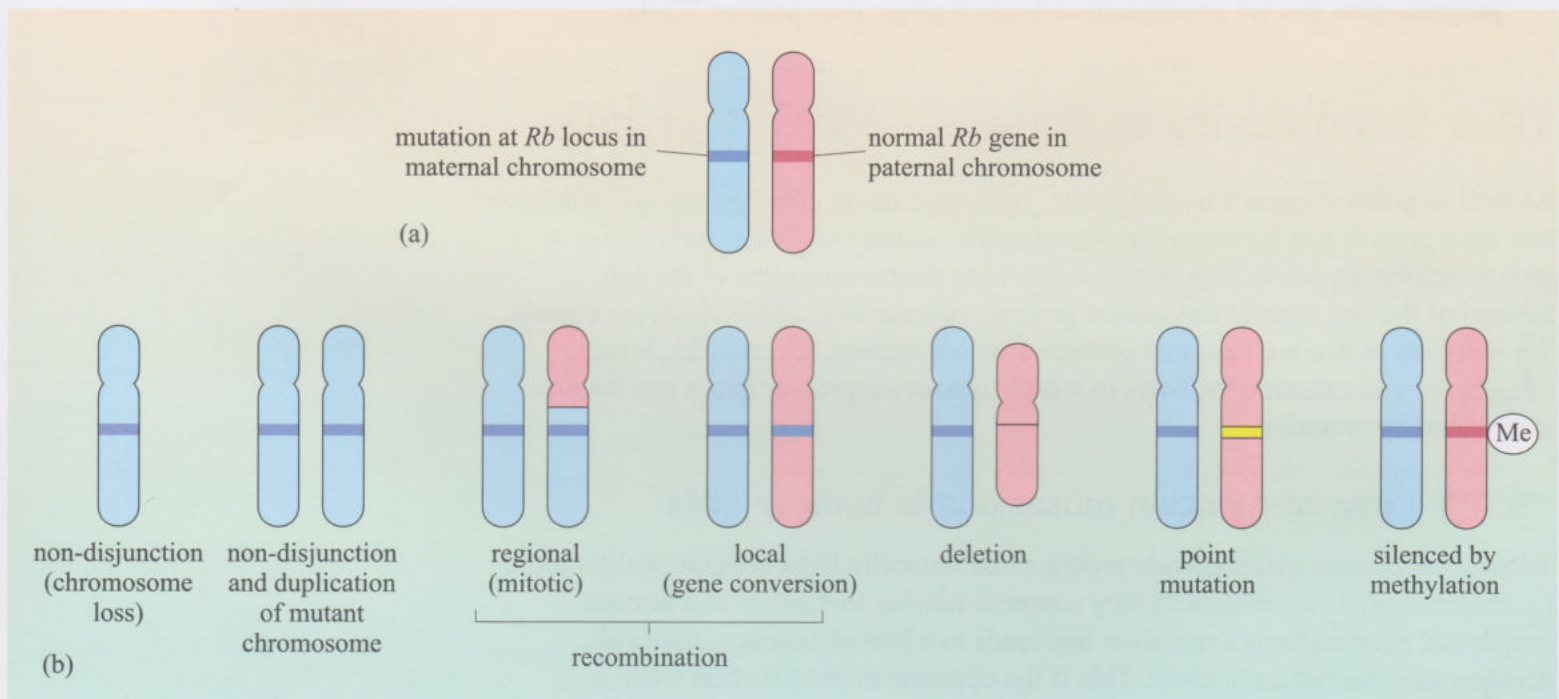


Figure 19.17 Following inheritance of a mutated copy of a tumour suppressor gene (here the *Rb* gene), there are several ways of losing the remaining, non-mutated copy on the other chromosome. These include chromosome loss with or without duplication, mitotic recombination or gene conversion, gene deletion (or attenuation), point mutation, gene silencing by methylation and other chromatin changes.

Box 19.5

Investigation of genomic DNA methylation in mammalian tumours

DNA methylation patterns and chromatin structure change during tumorigenesis. There is an overall genome-wide loss of DNA methylation, but there is also regional hypermethylation that is associated with gene silencing. Silencing of tumour suppressor genes will, of course, promote tumour development. For example, hypermethylation of the promoter of the *mutL* mammalian homologue *MLH1* and subsequent gene silencing can lead to DNA damage due to the absence of the encoded product, an essential protein for DNA repair mechanisms (see Sections 9.8.3 and 19.2.1).

Several techniques are available to screen tumour cells for altered methylation patterns. We will look at one of these, called restriction landmark genomic screening (RLGS). RLGS depends upon some restriction enzymes being sensitive to methylated cytosine residues within their recognition sequence. The enzyme NotI, for example, recognizes 5'-CG-rich sequences that occur rarely in the genome but will not cut if the cytosine bases in the restriction recognition sequence are methylated. In RLGS, a testgenome (e.g. that of a tumour cell) and a control genome are digested with NotI and the resulting DNA fragments compared. In regions of DNA with hypermethylation, as may be the case in genomic DNA from tumour cells, NotI sites will not be cut, and this will result in a different pattern of fragments between the normal and tumorigenic cell which are easily detected.

19.4.2 Rb and p53

The **retinoblastoma** gene (*Rb*) was the first tumour suppressor gene to be identified and was discovered by study of the rare human cancer, retinoblastoma. This tumour arises from neural precursor cells in the retina during development, and so is only found in children. Since both copies of a tumour suppressor gene need to be inactivated to produce a tumour, it can arise either by inheritance of a defective *Rb* gene and subsequent inactivation of the remaining functional copy in the retina, or (in children with no inherited defective mutations) by the very rare inactivation of both alleles in the same line of cells in the retina (see Figure 19.18). The clue to identifying the gene came from the observation that inheritance was associated with deletions on chromosome 13, where the *Rb* gene is located. The Rb protein acts as one of the main restraints on the cell cycle during the G1 phase, by binding to and inactivating transcription factors (such as E2F) required for the expression of the genes encoding cyclins A and E, thereby blocking entry into S phase (Section 8.3.4). The Rb protein must be especially important in restraining cell proliferation in the developing retina, although once the cells have reached maturity, Rb appears to be no longer critical. Loss of functional Rb protein has subsequently been found to be extremely common in several of the most common human cancers, underscoring its central role in tumorigenesis.

- ☐ How is Rb inactivated in normal cells?
- ☒ The cyclin D–Cdk complex inactivates Rb by phosphorylation (see Section 8.3.4).

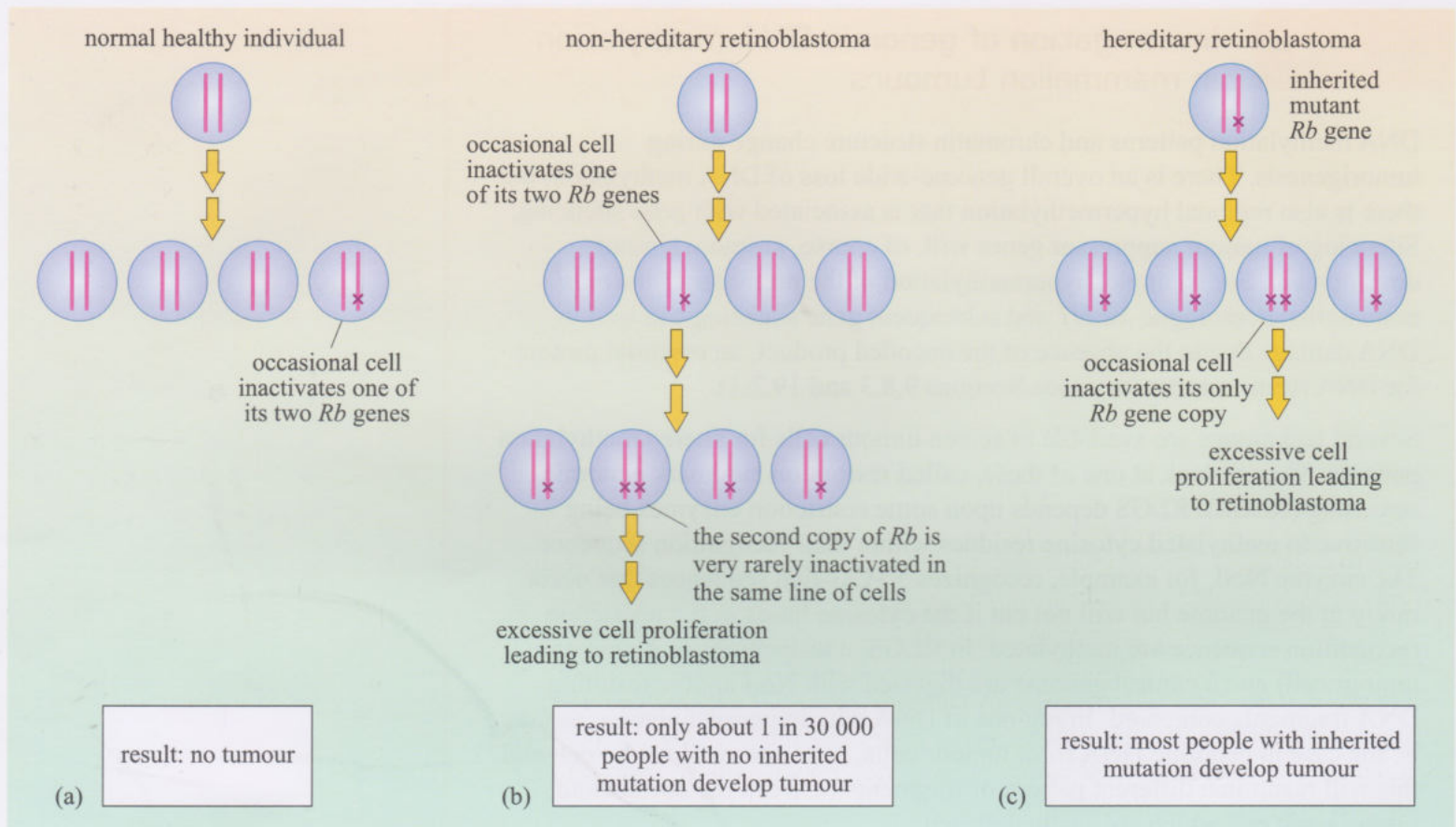


Figure 19.18 Retinoblastoma is caused by the inactivation of both alleles of the *Rb* tumour suppressor gene in the immature retina. (a) In a normal individual, occasional somatic inactivation of one *Rb* allele is of no consequence. (b) Only very rarely will that same cell line sustain inactivation of the second *Rb* allele and lead to retinoblastoma. (c) However, if a person has inherited an already mutant *Rb* allele, it is likely that the remaining allele will be inactivated during development and lead to retinoblastoma. Yellow arrows indicate successive rounds of cell division within a clone of cells.

The signalling pathway induced by transforming growth factor β (TGF β) prevents the phosphorylation of Rb. TGF β therefore maintains Rb in its active state and the cell cycle in check. TGF β thus acts as a growth-inhibitory factor in some cell types. Many human tumours have been shown to have downregulated or dysfunctional TGF β receptors (which are receptor serine–threonine kinases also implicated in mesoderm induction; Section 17.2.4). Moreover, the TGF β receptors phosphorylate and signal through a group of gene regulatory proteins called Smads (Table 17.1), which dimerize in the cytosol and translocate to the nucleus where they bind TGF β response elements upstream of target genes. One of these, Smad4, can also be mutated, similarly rendering the cell insensitive to the TGF β growth-inhibitory signal. We will meet the TGF β pathway again in the discussion of human colorectal cancer in Section 19.9.

Some DNA tumour viruses are able to interfere with the normal function of both Rb and p53 (see Figure 19.19). You will recall that p53 has already been discussed in Section 19.2 in relation to its central role in maintaining genetic stability, by arresting proliferation and allowing repair of DNA damage or by inducing apoptosis. It is clearly a very important tumour suppressor. Papillomaviruses are DNA viruses that cause warts in humans but are also associated with the development of cervical cancer. Their viral genomes encode two proteins that induce cell proliferation: E6, which can bind to and sequester p53, and E7, which can bind to and sequester Rb. Other DNA viruses, such as SV40 (a simian monkey papovavirus that has been

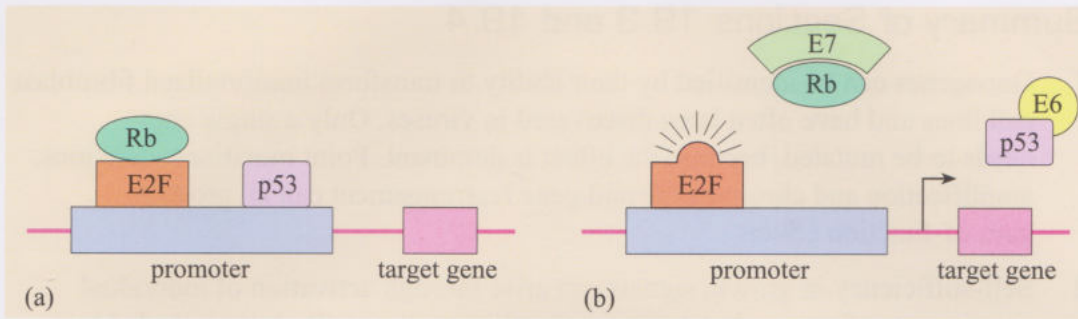


Figure 19.19 Some DNA viruses have proteins that can sequester Rb and p53. (a) Normally Rb binds to the transcription factor E2F and p53 binds to the promoter region, both of which prevent transcription of target genes. (b) Viral proteins E7 and E6 bind to Rb and p53 respectively, thereby lifting the block on transcription.

much used experimentally), encode one protein, the large T protein, which is sufficient to bind and sequester both Rb and p53. This strategy allows DNA viruses to escape the normal constraints of DNA replication in the host cell and enables them to use the cellular machinery to replicate their genomes freely, usually resulting in lysis of the cell and release of virus particles, which may go on to infect other cells. Occasionally, instead of this lytic cycle, a number of mechanisms, such as integration into the host genome, may cause these viral genes to become oncogenes, leading to the overproduction of proteins such as E6 and E7 or large T required by the virus for its replication. This in turn may lead to the long-term dysregulation of cell cycle controls in the host cell (Rb and p53), and predispose it to becoming tumorigenic. Box 19.6 shows how this knowledge is being exploited therapeutically to prevent abnormal cell growth.

Box 19.6 Gene therapy in human cancer

The delivery of a normal tumour suppressor gene to a cell to restore the function and/or expression of the protein product of a mutated gene has potential uses in the treatment of human cancer. This strategy is called gene therapy. A potential problem is that the therapeutic genes need to be introduced into all cancer cells in situ within the patient. Gene transfer technology is not yet capable of delivering the therapeutic gene to every tumour cell, so its use is currently limited to augmenting the efficacy of other therapies. The current method of choice for gene delivery is by inserting the gene of interest into the genome of a retrovirus that has been genetically manipulated so that it cannot replicate once it has infected a cell. Retroviral particles carrying the therapeutic gene infect at least 10% of all proliferating cells (including tumour cells). However, there are significant technical problems, relating to genetic rearrangement, to the limited amount of exogenous genetic information the retroviral particles can accommodate, and to the fact that they transfer the gene of interest permanently into the genome of the

target cell, possibly resulting in the activation/deactivation of other genes as they do so.

Another tactic is to introduce specific antisense DNA sequences (Box 5.2) or use RNA interference techniques (Box 10.5) to specifically block the expression of a mutated oncogene, such as *bcr-abl*. These approaches are currently undergoing clinical trials.

Recall that some DNA viruses induce the transformation of host cells by encoding proteins that bind to and inactivate p53. Mutated versions of these viruses have been developed to target transformed cells that lack p53. These mutated viruses lack the gene(s) for the p53-blocking protein(s) that the virus normally needs in order to replicate. In infected normal cells, viral replication will be prevented by the still-functional p53 and the cells will not undergo lysis. In infected tumour cells lacking functional p53, the virus will be able to proliferate, lysing and killing the cell. Thus, this defective virus offers a method for specifically destroying tumour cells lacking p53.

Summary of Sections 19.3 and 19.4

- 1 Oncogenes can be identified by their ability to transform immortalized fibroblast cell lines and have often been discovered in viruses. Only a single copy needs to be mutated, because the effect is dominant. Point mutations, deletions, amplification and chromosome and gene rearrangement can all produce gain-of-function effects.
- 2 Self-sufficiency in growth signals can arise through activation of individual components of a growth-promoting signalling pathway. Proteins encoded by proto-oncogenes include growth factors (e.g. PDGF), receptors (EGF receptors), cytoplasmic signalling molecules (Ras, Src and Bcr–Abl), and transcription factors (Myc).
- 3 Tumour suppressor genes, such as *Rb*, were originally discovered through study of rare inherited cancers. For cell tumorigenesis to occur, both copies need to be inactivated (e.g. by point mutation, deletion, methylation), which is less likely to occur than activation of a single oncogene allele, but has very profound effects on the cell.
- 4 Loss of tumour suppressor genes can cause insensitivity to either external growth-inhibitory signals, or to internal checkpoints. Loss of functional *Rb* causes deregulation of the G1/S checkpoint. Loss of p53 allows cells with sustained DNA damage to continue to replicate, when normally they would be arrested at entry into S phase, by p53/p21CIP-mediated mechanisms, until the damage was repaired.

19.5 Evasion of apoptosis

Up until now, we have discussed cellular tumorigenesis from the aspect of cell proliferation, i.e. mutations that give growth advantage by promoting cell division. However, dysregulation of *other* cellular processes may also increase the net cell population of a tumour. This may be achieved by a decreased rate of programmed cell death or apoptosis.

- ☐ Why should tumorigenic cells normally be triggered to undergo apoptosis?
- ☒ Because they have sustained widespread genetic damage.

A crucial step in the microevolutionary development of cells within a tumour is the occurrence of cell clones with mutations that cause them to evade apoptosis, despite cumulative widespread genetic damage. This may occur because the tumour acquires changes that allow it to evade apoptosis by either the intrinsic and/or extrinsic pathways.

- ☐ What will happen if a tumour cell evades apoptosis?
- ☒ It will continue to divide and hence propagate its damaged genome through subsequent cell generations.

Resistance to apoptosis can be acquired by tumour cells by a variety of mechanisms. One such strategy is to inactivate pro-apoptotic signals. We have already described the multiple roles of p53 in pathways arresting proliferation or triggering apoptosis

in cells with DNA damage. There are several other apoptotic pathways that can become dysregulated during the process of tumorigenesis, notably those mediated by members of the Bcl-2 family of proteins (Section 14.4.4).

- ☐ What is the normal cellular role of the Bcl-2 family of proteins?
- ☒ The balance between pro- and anti-apoptotic Bcl-2 family members determines whether mitochondrial membrane permeability is increased and so whether the intrinsic apoptotic pathway is activated.

Recall from Chapter 14 that the *bcl-2* gene was originally identified in a B-cell lymphoma. A common translocation event in these tumours results in the *bcl-2* gene being placed under the control of the active promoter of another gene, which drives its overexpression. The role of Bcl-2 itself in normal cells is to inhibit apoptosis; therefore Bcl-2 overexpression will cause the survival of cells that otherwise should have been eliminated by apoptosis.

The intrinsic apoptosis pathways that would normally lead to 'death by neglect' can be bypassed in some tumours in which evasion of apoptosis is achieved by interfering with anti-apoptotic pathways elicited by survival signals. For example, the cytokine IGF-1 is required for survival of a number of different types of cell, and lack of IGF-1 leads to apoptosis. (The IGF-1 signalling pathway is shown in Figures 18.9 and 19.24.) The pathway is dysregulated in a number of different types of tumour, including breast and prostate cancer. Moreover, the inositol phospholipid phosphatase PTEN, which normally blocks the IGF-1-initiated survival signal pathway, has been identified as a tumour suppressor. This is because PTEN antagonizes the action of PI 3-kinase in normal cells by removing phosphate groups of phosphatidylinositol second messengers (Section 13.3.2). As a result, PTEN in normal cells blocks the IGF-1 survival signal pathway which is mediated by PI 3-kinase and PKB/Akt activation. Inactivation of both alleles of the *PTEN* tumour suppressor gene is almost as frequent in tumours as that of p53 (PTEN mutations have also been implicated in the invasive potential of tumour cells; Section 19.8).

As well as disruption of the intrinsic apoptotic pathways, the extrinsic pathway can also be dysregulated in tumour cells.

- ☐ How is the extrinsic pathway of apoptosis initiated?
- ☒ Binding of ligands (e.g. Fas death ligand) to cell surface receptors (e.g. Fas) and activation of downstream effectors such as caspases (e.g. caspase 8-mediated activation of caspase 3 and Bid).

The Fas pathway has been found to be dysfunctional in tumour cells derived from certain human lung and colorectal tumours.

- ☐ What kind of change in the extrinsic pathway would promote tumour growth?
- ☒ Changes that may bypass the Fas pathway. These include loss of function of receptors such as Fas or components of the DISC, or of key caspases such as caspase 8.

The most common mechanism by which the Fas pathway is bypassed in lung and colorectal cancer involves the expression of a decoy receptor for the Fas ligand, which is upregulated and does not signal, thus blocking the 'real' death signal.

Apoptosis appears to be the major barrier to cancer that must be circumvented to progress from abnormal cell growth to rapidly growing tumours. The importance of the apoptosis pathways in controlling tumour development is illustrated in fibroblasts that express Myc. One would normally expect such cells to have a high rate of proliferation, but Myc-expressing fibroblasts cultured without serum or growth factors have a high rate of apoptosis. However, if in addition the *bcl-2* gene is overexpressed, apoptosis is prevented and Myc-expressing fibroblasts show increased proliferation. These data suggests that overexpression of oncogenes often triggers apoptosis *in vivo*, thereby eliminating dysregulated cells with susceptibility for abnormal growth. Oncogene-induced apoptosis might constitute a normal physiological defence mechanism against the formation of tumours and, therefore, the requirement for both increased cell proliferation and a failure of apoptosis may explain why cancer is not a more common occurrence than it is.

19.6 Limitless replicative potential

We have established that three acquired cellular properties are essential for tumorigenesis—self-sufficiency in growth signals, insensitivity to growth-inhibitory signals and resistance to apoptosis. However, most human somatic cells have a pre-programmed limit to the number of times they divide – human fibroblasts, for example, will generally divide 25–50 times in culture, then stop. Cells within tumours are also limited in their growth abilities by a finite replicative potential and usually do not grow into larger-size tumours unless the mechanisms leading to replicative senescence are overcome.

- ☐ What events are associated with such replicative senescence?
- ☒ Shortening of telomeres, DNA damage, decondensation of chromatin, overactivation of some mitogenic stimuli, and of some oncogenes (see Figure 18.5).

Replicative senescence appears to be due, at least in part, to the progressive shortening of telomeres with each round of replication of chromosomal DNA (in the absence of telomerase).

- ☐ What happens to a chromosome when its telomeres shorten to a critical length?
- ☒ Further rounds of replication could cause the erosion of genetic information through loss of terminal DNA. Chromosome damage could also be caused as a result of the DNA ends being recognized as DNA breaks which would be repaired by the cellular machinery, resulting in chromosome rearrangements.
- ☐ How would a cell normally respond to chromosomal damage that might result from the shortening of telomeres?
- ☒ DNA ends with short telomeres would be detected as double-strand breaks by DSB-specific proteins which would trigger ATM activation and p53-mediated cell-cycle arrest as described in Section 19.2 (and in Chapters 9 and 18).

If such a cell had sustained p53-inactivating mutations, instead of entering replicative senescence the cell would continue to divide uncontrollably. However, continued rounds of replication in this circumstance will produce tumour cells with ever more truncated chromosomes and severe genetic instability. How can telomere

loss result in genetic instability in p53-deficient tumour cells? If p53 is lost, a cell with critically short telomeres may enter S phase and progress through the cell cycle in spite of DNA damage. The DNA ends of sister chromatids may first fuse and then break during mitosis, creating two aberrant chromosomes with no telomeres, allowing several rounds of fusion–breakage (Figure 19.20). In a developing mass of tumour cells, this cycle of fusion–breakage will result in many different cells with different combinations of mutations and degrees of genetic and chromosomal instability.

You can see how the loss of telomere repeats can induce chromosomal instability, but most tumours are highly proliferative and chromosomal damage may be too severe for cells to be viable. The way many tumour cells solve this problem is by reactivating the expression of the telomerase enzyme. Human telomerase, which lengthens telomeres in germline cells and some stem cells, is normally at low levels or absent in the majority of human somatic cells (see Chapters 9 and 18). A mutant cell that expresses telomerase, and so maintains its telomeres, may escape replicative senescence and have an immediate competitive growth advantage by comparison with its senescing neighbours. It appears from study of human tumours, that telomerase *is* reactivated in approximately 85% of them. However, in human breast and colorectal cancer, for example, it is often reactivated at a fairly advanced stage of tumour development, only *after* gross chromosomal changes have already occurred. This can be explained if p53 is inactivated first, leading to massive genetic instability (as in Figure 19.20).

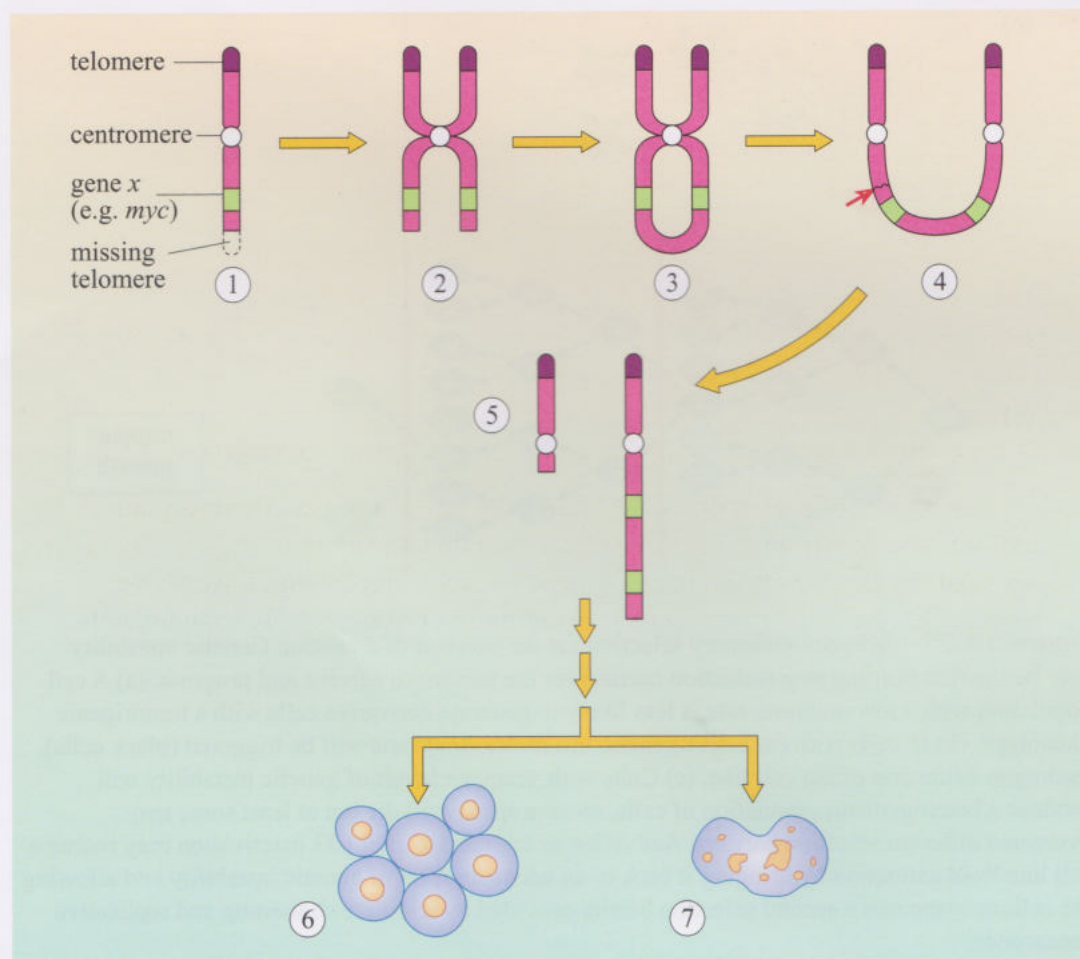


Figure 19.20 Reactivation of telomerase in cells that have lost p53 enables cells to maintain a more viable level of chromosomal (in)stability, allowing the cells to progress to an enlarged tumour rather than dying of massive chromosomal damage. A cell that has lost a telomere (1), and has lost p53 can still enter S phase and replicate, despite the DNA damage (2). Absence of telomeres may lead to fusion of the sister chromatids (3), with the potential for further chromosomal damage, e.g. one daughter cell may end up with a chromosome that has two copies of a gene such as *myc*, and the other daughter cell will have a shortened chromosome lacking the *myc* gene (4, 5). (The red arrow denotes the break point.) If a cell acquires mutations that increase telomerase activity, the restoration of the ends of the chromosomes can limit the extent of further damage so that the cell can survive (6), whereas without telomerase, the massive chromosomal instability would lead to cell death (7). Activation of telomerase after p53 inactivation may rescue a cell line from extinction by avoiding excessive chromosomal instability.

In most cases, cells with excessive genetic instability will undergo apoptosis. In other cells, however, a mutation may lead to an increase in telomerase activity which will allow these cells to continue to divide and produce heterogeneous populations of cells (Figure 19.21). Whereas in cells with low genetic instability the low mutation rate would not allow them to produce a tumour, excessive genetic instability would lead to extinction of the cell line. Increased telomerase activity therefore becomes a selection barrier for the progression of tumours, and telomerase inhibitors may have therapeutic uses, as discussed in Box 19.7.

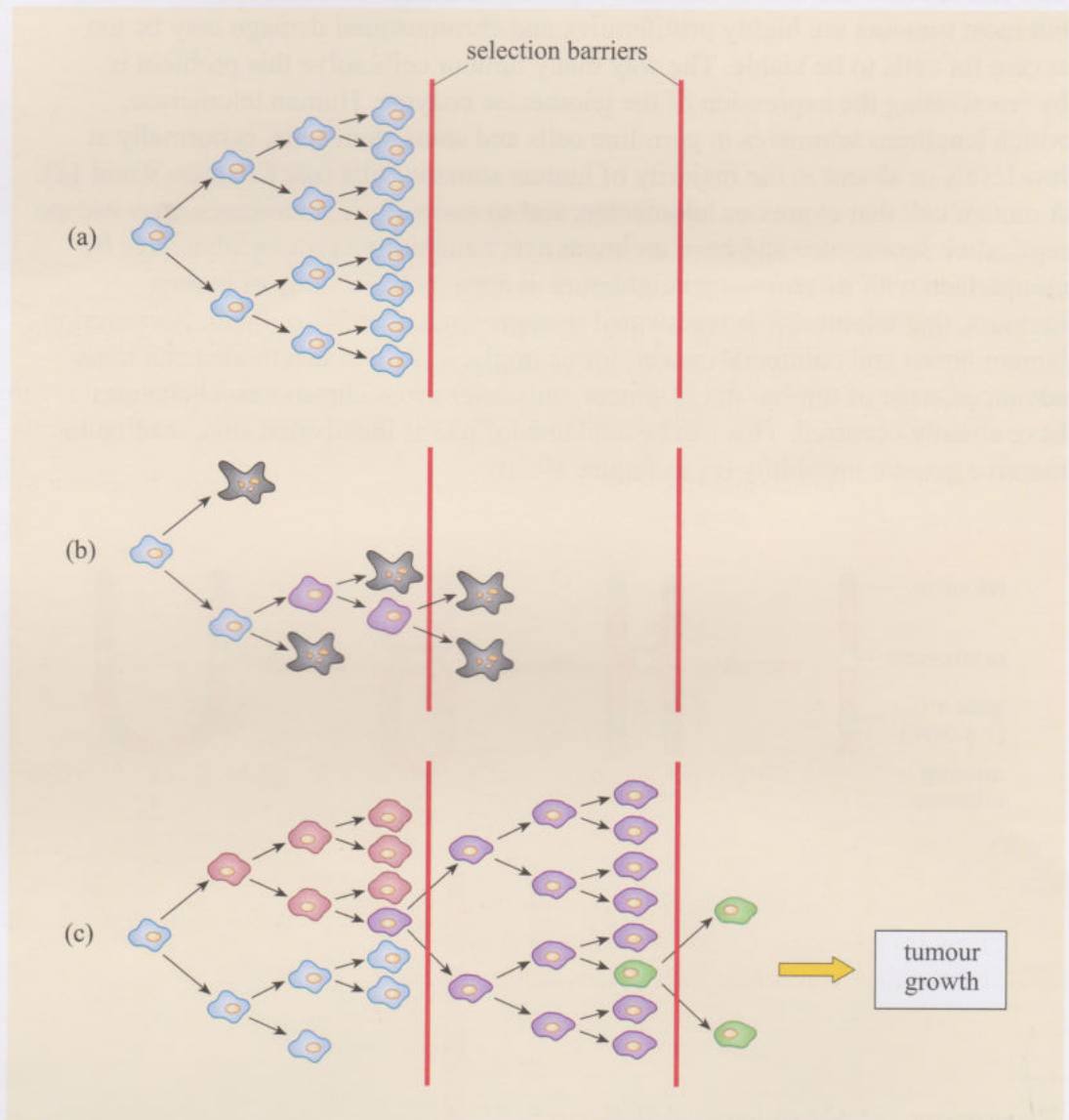


Figure 19.21 Microevolutionary selection for the survival of a tumour. Genetic instability may be the first limiting step (selection barrier) for the tumour to survive and progress. (a) A cell population with a low mutation rate is less likely to generate derivative cells with a tumorigenic phenotype. (b) In cells with excessive genetic instability, apoptosis will be triggered (black cells), leading to extinction of the cell line. (c) Cells with adequate levels of genetic instability will produce a heterogeneous population of cells, most progeny will die but at least some may overcome different selection barriers. Activation of telomerase after p53 inactivation may rescue a cell line from extinction by bringing it back to an adequate level of genetic instability and allowing the cells to overcome a second selection barrier provided by telomere shortening and replicative senescence.

Box 19.7 Telomerase activity inhibitors in human cancer

There are various approaches under investigation for targeting telomerase in human tumours. We will look in turn at the use of antisense technology, at reverse transcriptase inhibitors and at molecules targeting the telomeric G-rich overhang.

Antisense and RNAi approaches

Antisense and RNAi technology were introduced in Box 5.2 and Box 10.5 respectively. They are targeted at specifically blocking telomerase expression. We will consider here antisense technology.

- ☐ What unique nucleic acid feature associated with telomerase would be a possible target of antisense therapy? Why?
- ☒ The 11 base pair region of the RNA template of telomerase. Without the template no new telomeric DNA can be synthesized.

Downregulation of telomerase-catalysed extension of telomeres has been achieved with appropriate antisense oligonucleotides, but the use of oligonucleotide therapy is hampered by problems of low cellular uptake and the instability of antisense DNA. Antisense DNA instability can be partly overcome by modifying the oligonucleotide's sugar-phosphate backbone, but cellular uptake remains a major obstacle for these types of compounds.

Reverse transcriptase inhibitors

Azidothymine (AZT) is an anti-retroviral drug which inhibits reverse transcriptase and is used to combat HIV infection. It has also been tested as a telomerase

inhibitor. However, observed changes in cell proliferation could not be attributed to telomerase inhibition alone, since AZT is also a known DNA polymerase inhibitor.

Targeting the G-quadruplex structures

Recall from Chapter 5 that the terminal telomeric G-rich repeats are single-stranded and can spontaneously adopt a quadruplex structure. The existence of quadruplex DNA has not yet been confirmed *in vivo*. However, many proteins have been found to selectively bind to quadruplex DNA, suggesting it has a role *in vivo*. Telomerase is only active on the non-structural single-stranded telomere DNA, which in human cells can be up to 200 nucleotides in length.

- ☐ Based on this information, can you suggest what kind of molecule might inhibit telomerase activity?
- ☒ Any molecule that specifically removes single-stranded segments of DNA, by stabilizing secondary structures, such as quadruplex DNA.

The design of G-quadruplex-interacting ligands has stemmed from experience with DNA intercalators such as ethidium bromide derivatives. Clearly though, the ligands selected must bind with much greater affinity to quadruplex DNA than to duplex DNA to avoid secondary toxicity. Porphyrins (TMPyP2 and 4) constitute another example of drugs that have been extensively tested and found to inhibit telomerase by binding quadruplex DNA.

Summary of Sections 19.5 and 19.6

- 1 Dysregulation of the pathways of apoptosis is as important in the microevolution of tumours as the changes that increase the rate of cell proliferation. Tumour cells acquire mutations that cause them to resist apoptotic signals by either inhibiting pro-apoptotic signals or activating anti-apoptotic pathways. Both intrinsic and extrinsic pathways can be disrupted in tumorigenic cells.
- 2 Tumour cells acquire mutations that cause them to avoid replicative senescence. One mechanism involves p53 inactivation, so that cells with damaged telomeres continue to divide. Tumour cells may also reactivate telomerase, often after significant chromosomal damage has been sustained, thus allowing clonal tumour cells that would otherwise be non-viable, to survive and proliferate.

19.7 Sustained angiogenesis

In order to allow nutrients to reach its constituent cells, a solid tumour must induce the *de novo* formation of blood vessels (**angiogenesis**) and lymphatic vessels (**lymphangiogenesis**) that penetrate the solid cellular mass. Normal cells within tissues are usually within 100 μm of a capillary so that diffusion of essential oxygen and nutrients from the bloodstream is not a limiting step for cellular metabolism. Most tumour cells originally lack the ability to induce angiogenesis, but the acquisition of angiogenic properties allows the tumour to progress to a larger size. This is because tumour cells, especially in areas within the core of the tumour, must obtain adequate blood supply, or suffer regional necrosis. Some tumours have particularly high growth rates and these have an even greater need to maintain an adequate blood supply. In addition to ensuring tumour cell metabolism, penetration by blood vessels and lymphatics probably facilitates the escape of tumour cells from within the original tumour solid mass into the blood and lymphatic system to form metastases in other tissues/organs, as we shall see in Section 19.8.

Angiogenesis is regulated by a fine balance between angiogenic and anti-angiogenic signals. Many different growth factors have been implicated in angiogenesis; an important example is **vascular endothelial growth factor (VEGF)**. VEGF is synthesized and secreted by most cell types in response to hypoxia. Endothelial cells expressing VEGF receptors (a receptor tyrosine kinase, like those described in Section 13.2.2, p. 98) on the cell surface respond by migrating towards the source of VEGF, proliferating and then differentiating to form new capillaries. Almost every type of human solid tumour has been shown to express VEGF (and often in elevated amounts), so it is likely that tumour cells maintain their blood supply by this mechanism.

The significance of an adequate blood supply to tumour growth and survival was illustrated by a classic tumour transplant experiment carried out in the 1970s. When transplanted into rabbit corneal tissue (which is normally avascular, i.e. it has no blood supply), human tumours would initially only grow to a maximum of 2 mm. But once they had induced growth of blood vessels from the surrounding tissue, they could proliferate rapidly.

Expression of endogenous inhibitors of angiogenesis such as thrombospondin and endostatin may also be inhibited in some tumours. The mechanism by which the balance in the expression of angiogenic regulators is shifted is not clear, although some mutations already acquired by tumour cells may be involved. For example, in cell transfection experiments it has been shown that activated oncogenic Ras upregulates VEGF expression whereas p53 overexpression upregulates the production of thrombospondin (therefore loss of p53 may be related to decreased levels of this angiogenesis inhibitor). Anti-angiogenic drugs may have potential in combating human solid tumours, and are discussed in Box 19.8.

The evidence that tumours are able to direct the activity of endothelial cells has led researchers to recognize the complex interactions between tumour cells and other cell types. It has been postulated that normal cells may even be exploited by tumour cells to promote their own proliferation. For example, fibroblasts and immune cells surrounding tumour cells have been shown to produce extracellular matrix proteins and growth factors that support tumour cell growth. The view that considers tumours a mere mass of clonally derived cells is being replaced by another that regards tumours as complex tissues with diverse cell types, some mutant and some normal, but all interacting with each other to produce a distinctive cellular community, as shown in Figure 19.22.

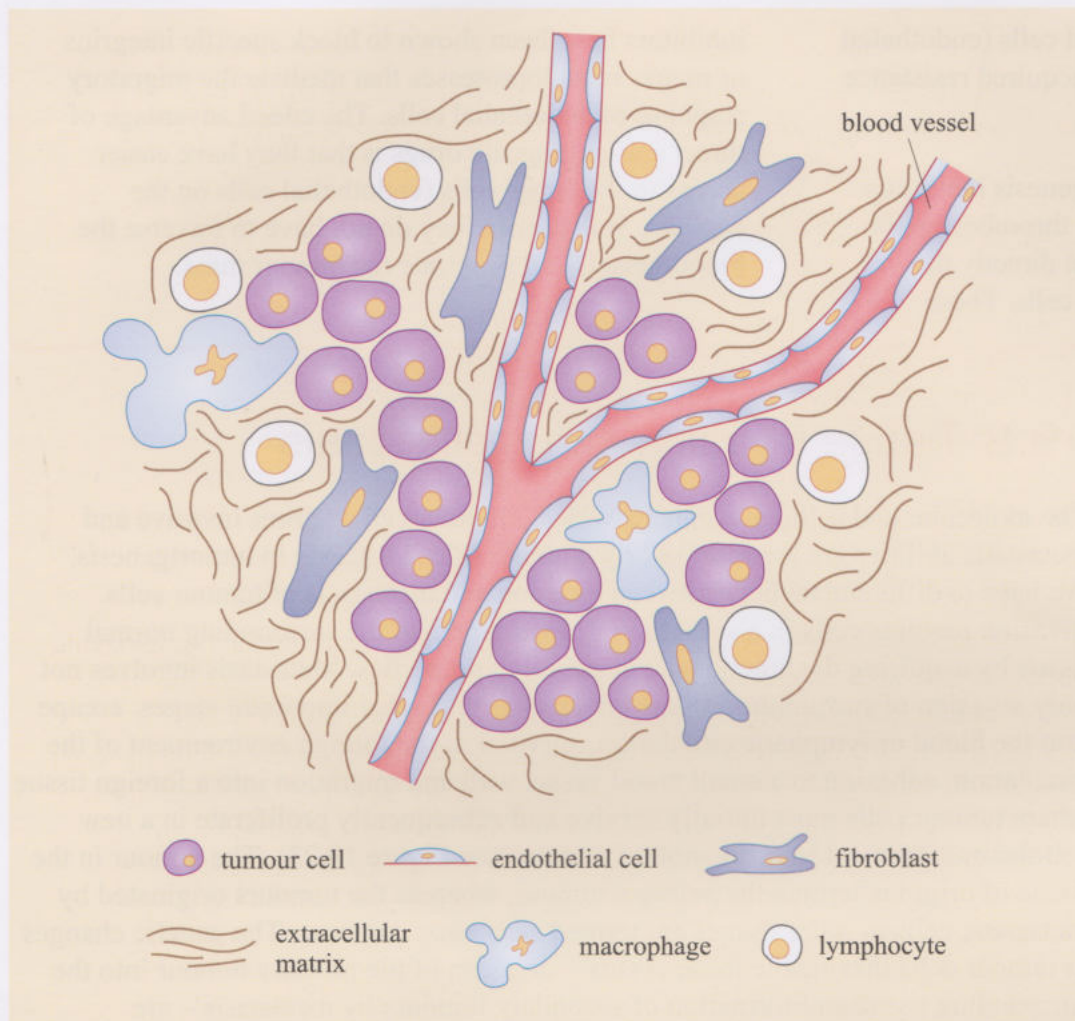


Figure 19.22

Tumours are increasingly viewed as an interacting complex of normal and tumour cell types. In this diagram, tumour cells are shown to interact with fibroblasts, lymphocytes, macrophages and endothelial cells as well as with the extracellular matrix.

Box 19.8 Angiogenesis inhibitors in human cancer

A novel anti-tumour approach is to target the growth of the endothelial cells that form the blood vessels of the developing tumour, rather than targeting the tumour cells directly. There are two basic approaches to inhibiting angiogenesis. The first is to target specific angiogenic signals by using antibodies to neutralize angiogenic proteins (e.g. anti-VEGF antibodies), soluble non-signalling forms of receptors to angiogenic signals (e.g. soluble truncated forms of VEGF receptors which only contain the extracellular ligand-binding region) or molecular inhibitors of signal transduction (e.g. inhibitors of the tyrosine kinase activity of the VEGF receptor).

- ☐ Can you suggest any limitations of using specific therapies such as antibodies targeted to a single angiogenic factor?
- ☒ The tumour may produce additional angiogenic factors other than VEGF.

This is indeed the case in many human breast cancers. In early stages, 60% of breast tumours produce VEGF exclusively, but in later stages of tumour development most human breast cancers produce a complex range of angiogenic factors including PDGF and FGF.

The second approach is to devise therapies that inhibit angiogenesis directly, i.e. endothelial cell proliferation and migration, regardless of the angiogenic signals being produced by the tumour.

- ☐ What advantage might direct angiogenesis inhibitors have over specific inhibitors of angiogenic signals (e.g. VEGF)?
- ☒ Angiogenesis inhibitors will inhibit endothelial cell proliferation and migration regardless of which angiogenic factor is being produced by the tumour cells. They also have the related advantage that

because the drug targets normal cells (endothelial cells) instead of tumour cells, acquired resistance should not be a problem.

Drugs based on endogenous angiogenesis inhibitors such as the peptides endostatin and thrombospondin are examples of therapies that target directly the angiogenic response of endothelial cells. These

inhibitors have been shown to block specific integrins or matrix metalloproteases that mediate the migratory response of endothelial cells. The added advantage of direct anti-angiogenic drugs is that they have easier access to the target cells (endothelial cells on the capillary wall), since they do not have to traverse the blood vessels and penetrate the tumour mass.

19.8 Tissue invasion and metastasis

The molecular and cellular events by which a tumour cell acquires invasive and metastatic abilities are the most poorly understood of all stages in tumorigenesis. We have to differentiate here between invasion and metastasis of tumour cells. Invasion requires cells in a solid tumour to escape into the surrounding normal tissue by acquiring dysfunctional cell adhesive properties. Metastasis involves not only invasion of surrounding tissue but also several other important stages: escape into the blood or lymphatic circulation, survival in the foreign environment of the circulation, adhesion to a small blood vessel wall and migration into a foreign tissue where tumour cells must initially survive and subsequently proliferate in a new cellular environment to form another tumour (see Figure 19.23). The tumour in the tissue of origin is termed the *primary* tumour, whereas the tumours originated by metastatic cells in other tissues are termed *secondary* tumours. The genetic changes in tumour cells that enable these events – invasion of the primary tumour into the surrounding tissue and formation of secondary tumours by metastasis – are probably highly dependent on the tissue of origin of the primary tumour, on previous genetic lesions acquired by the tumour cells, and on the tissue where metastasis occurs.

We have seen from Chapters 13–16 that normal cells can only survive and proliferate if they constantly receive a spectrum of growth and survival signals particular to their cell type. These include soluble growth factors, usually secreted in a paracrine fashion by nearby cells within the tissue.

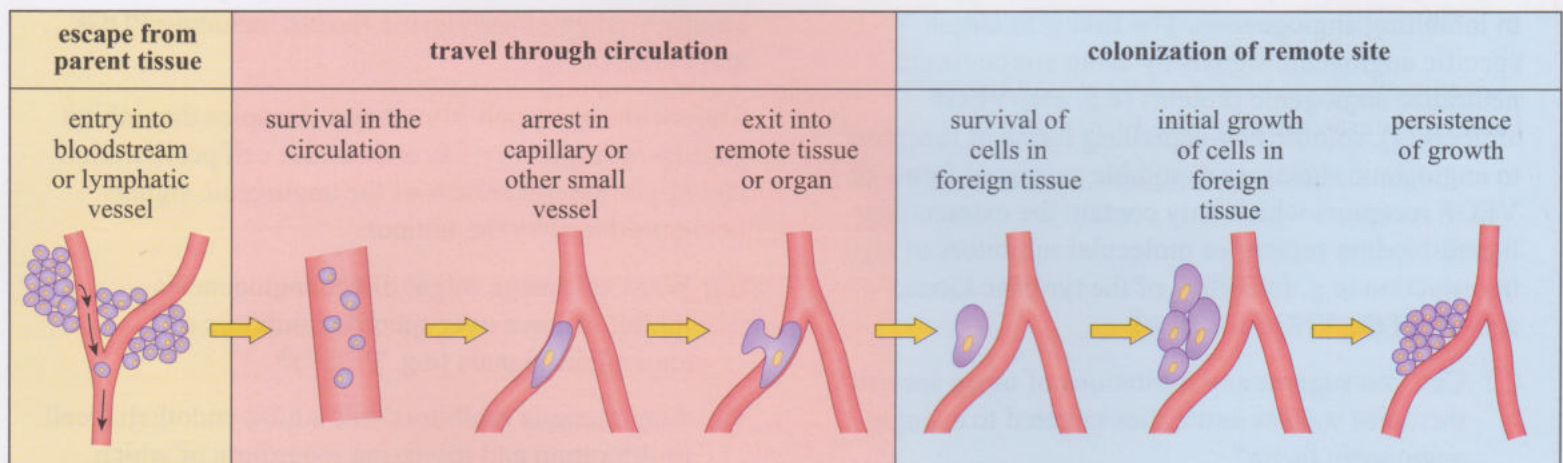


Figure 19.23 There are many barriers to metastasis. Using labelled tumour cells, it is possible to show which stages of the metastatic process are limiting, i.e. where large numbers of tumour cells are lost. Escape from the parent tissue and establishment in the remote tissue appear to be the most limiting stages.

- ☐ In addition to soluble factors, what other extracellular interactions determine growth and survival of cells within tissues?
- ☒ The direct interactions between adjacent cells and between cells and the extracellular matrix (ECM) by means of adhesion molecules. Cadherins, Ig superfamily CAMs and a subset of integrins mediate cell–cell adhesion, whilst integrins are the main receptors mediating cell–ECM adhesion (Chapter 16).
- ☐ What properties must be acquired by cells of the primary tumour in order to achieve invasiveness of the surrounding tissue?
- ☒ The normal interactions between adjacent cells and between cells and the ECM must be disrupted.

Cell–cell and cell–ECM interactions anchor, orientate and organize the cytoskeleton of cells. They can also, especially in the case of integrins, activate growth-promoting intracellular signalling pathways. Invasive tumour cells appear to disregard normal, tissue-specific, interactions and they are able to break through tissue structure and colonize the surrounding tissue. The expression of E-cadherin, an adhesion molecule that normally functions to hold adjacent cells of epithelial origin together (Section 16.2.1), is lost in some human breast and stomach carcinomas. Introducing the *E-cadherin* gene back into these cells in culture makes them more adhesive and therefore less invasive.

- ☐ Can you think of an alternative strategy by which a tumour cell may acquire a similar function to that provided by loss of E-cadherin?
- ☒ A mutation resulting in the inactivation or loss of a catenin, such as β -catenin, an anchoring protein that mediates the interactions between E-cadherin and the actin cytoskeleton.

Other adhesion molecules involved in invasion and metastasis of tumour cells are integrins, although due to the number of different types of different α and β integrin subtypes, their exact role is less well established. Some tumour cells of carcinomas have been shown to shift the expression from integrins that favour interactions with the ECM in normal tissue to those that interact with degraded ECM components.

- ☐ What mechanism, also used by migrating leukocytes, would enable tumour cells to migrate through the ECM?
- ☒ The secretion of extracellular proteases which break down local areas of ECM and so enable migration of tumour cells.

Proteases involved in the migration of tumour cells are mainly matrix metalloproteases (MMPs) or serine proteases and are usually bound to receptors on the cell surface.

- ☐ How is the action of these proteases normally limited?
- ☒ By the secretion of protease inhibitors. These include two types of tissue inhibitors of metalloproteases: TIMPs (MMP inhibitors) and serpins (serine protease inhibitors).

Some human tumours have been found to secrete extracellular proteases and there is interest in developing therapeutic protease inhibitors.

Once the tumour cells have escaped into the circulation, their destination is initially determined by anatomy. Cells escaping into the blood will travel first in the vascular system until they become lodged in the next capillary bed within another tissue. The most likely organs where this would happen will be the liver or the lungs, due to the large volumes of venous blood delivered to these first-pass organs. Tumour cells escaping into lymphatic vessels are likely to be arrested in the draining lymph nodes where they can invade the surrounding tissue and enter the blood circulation.

Metastasis is a very inefficient process. For example, it has been shown in blood samples taken before surgery for primary human renal carcinoma, that over 10^7 tumour cells are released into the circulation every day. Obviously then, the vast majority of cells that enter the bloodstream never manage to colonize another tissue. Because of the heterogeneity of different clones of tumour cells, it is likely that only a subset of the cells that reach the blood or lymphatic vessels also have the necessary changes to allow them to access and survive in other tissues. The cells that do, may have a reduced requirement for survival and growth signals from the host tissue. It is uncertain whether some tissues are more susceptible to colonization by metastatic cells than others. As discussed above, anatomy obviously dictates the proportion of cells that are deposited in different tissues. The most common initial sites of metastases in humans are the lung, liver and lymph nodes (as discussed above), but also bones and brain. Yet anatomy does not always explain the distribution of secondary tumours and it is possible that some tissues are more supportive of tumour cell growth than others by, for example, expressing appropriate growth factors and compatible extracellular matrix proteins. However, the search for genes that are *specifically* expressed by tumour cells with metastatic behaviour has proved difficult. Studies using cDNA expression arrays have shown that metastatic tumour cell lines express high levels of metalloproteases and of a Rho-family small G proteins, but this is also the case for other tumour cells that do not metastasize. Loss of the tumour suppressor gene *PTEN* is a molecular marker of metastatic cells in melanoma, prostate carcinoma and glioblastoma, although *PTEN* inactivation occurs early in tumour evolution and is also widely present in cells of the primary tumour (and metastasis is a late event). During the lifetime of this course, it is probable that this area of cancer research will become much clearer.

Summary of Sections 19.7 and 19.8

- 1 Solid tumours secrete VEGF and other angiogenic factors which induce angiogenesis.
- 2 Tissue invasion by tumours requires the breakdown of normal cell–ECM and cell–cell adhesion interactions; for example, E-cadherin is absent in some tumours.
- 3 Metastasis is very inefficient and poorly understood. The cells need to break through the basal lamina of blood or lymphatic vessels, probably aided by the secretion of serine proteases or matrix metalloproteases. Once in the circulation, they lodge in capillary beds (or if in lymphatics, lodge in draining lymph nodes) and may then colonize the new tissue. Successful colonization probably depends on the degree of self-sufficiency of the metastatic cells.

19.9 From tumour cells to cancer

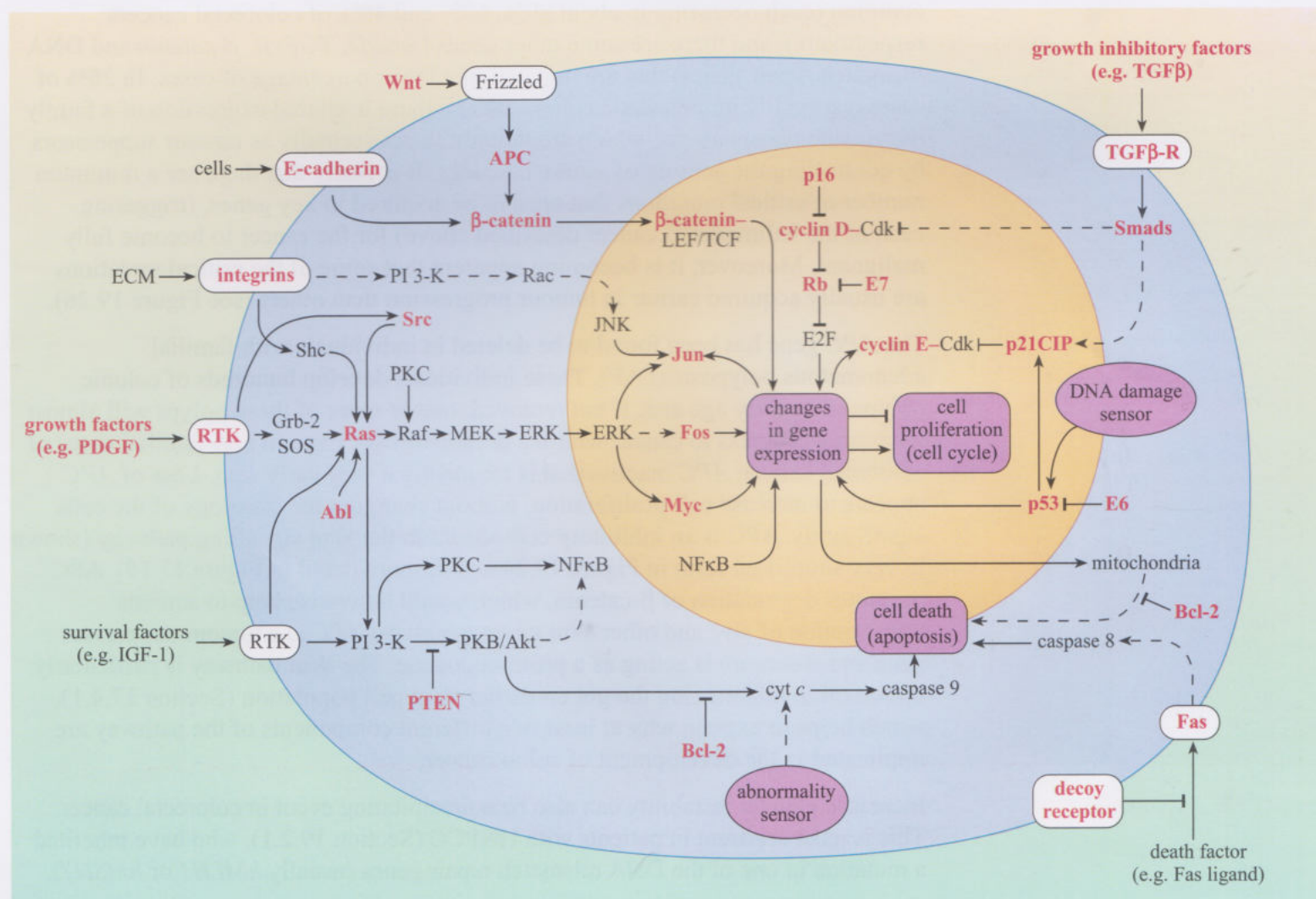
19.9.1 The molecular pathways to human cancer

We will summarize here the molecular mechanisms, discussed in this chapter, by which a cell may become tumorigenic. Figure 19.24 illustrates some of the major proteins that can be dysregulated in human cancer, in the form of an integrated cell circuitry. You are not expected to commit it to memory, but it will enable you to appreciate how many different pathways can be dysregulated in cancer.

The molecular pathways by which normal cells acquire a tumorigenic phenotype, eventually progressing to human cancer, are extremely diverse. Even within a given type of human cancer, a clonal population of tumour cells may show loss of p53 while another may express an activated *ras* oncogene, even though morphological differences between the two cell clones are undetectable. Moreover, the onset of mutations in specific oncogenes and/or tumour suppressor genes, whether early in some tumours or late in others, may determine the stage in which other tumorigenic properties – evasion of apoptosis and replicative senescence, angiogenesis, invasion and metastasis – are acquired. Yet all human cancers with progressing degrees of malignancy share these biological endpoints, independently of how they are ultimately reached. We shall consider now a type of human cancer, colorectal cancer, which progresses in an orderly manner, a feature that has advanced greatly our understanding of tumorigenesis.

Figure 19.24

A molecular circuit diagram, illustrating some of the major proteins that can be dysregulated in cancer (shown in bold red type). The diagram is highly simplified, concentrating mostly on the pathways discussed in this and previous chapters.



19.9.2 Human colorectal cancers as a model of tumour progression

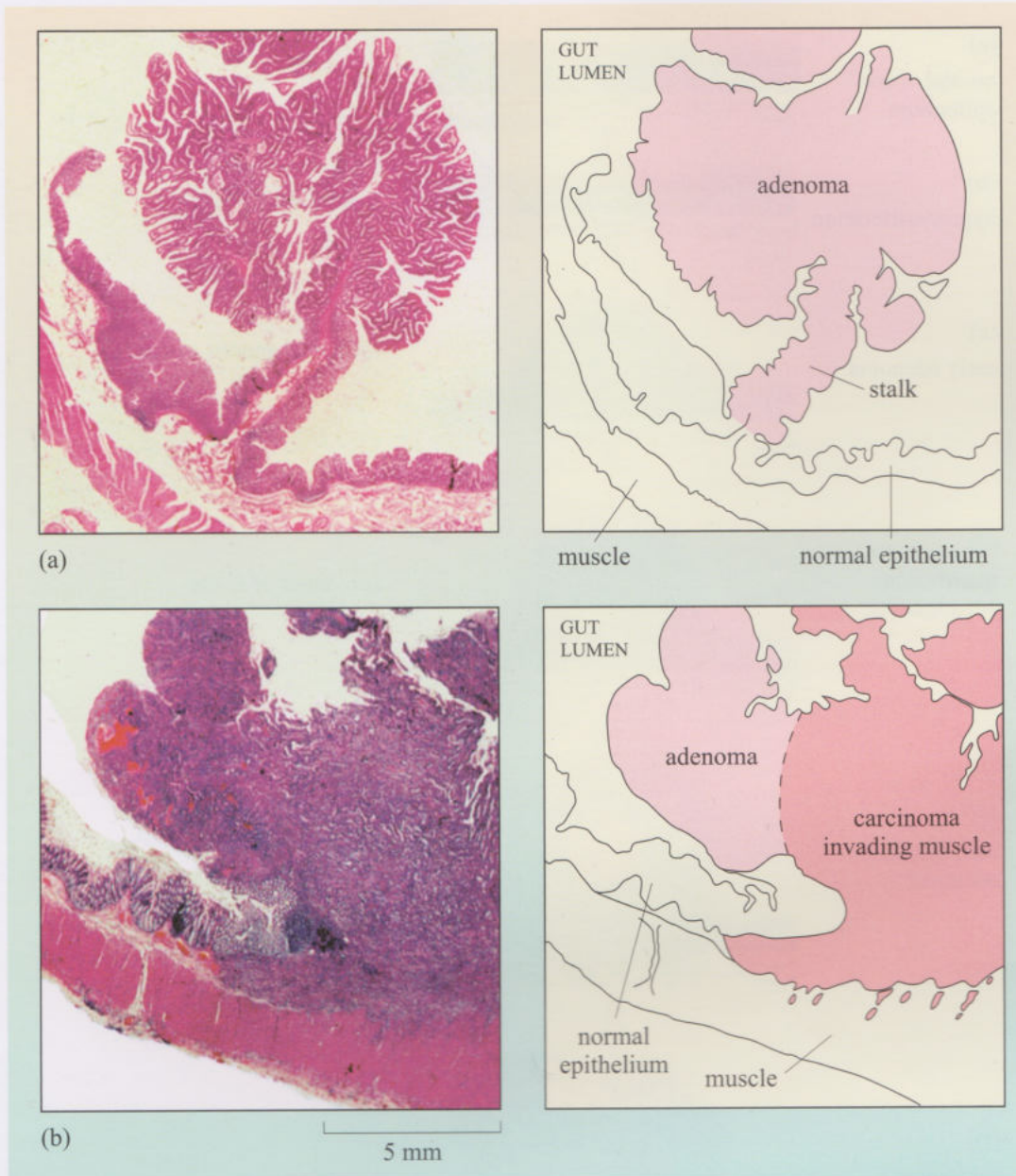
Now that we have described the main characteristics of tumour cells and the genetic lesions underlying them, we can see how they operate in a common class of human tumours – colorectal cancers – that have a relatively reliable pattern of progression. Colorectal cancers cause 60 000 deaths (i.e. about 11% of cancer deaths) per year in the USA. The disease mostly manifests itself in later life. However, examination of colon tissue samples from individuals in their fifties onwards can reduce mortality statistics by up to 75%. This is because the tumour begins as an easily identifiable benign polyp that may take several decades before turning malignant. Surgical removal of the polyp is simple and highly effective which makes the investigation of the pathogenesis of colorectal cancer from a very early stage relatively easy.

Small polyps less than 1 mm across contain relatively normal-looking cells. The larger the polyp, the more abnormal and undifferentiated the cells appear, and it is possible to see one or more subpopulations of cells, which are presumably mutant subclones. Later, invasion of the epithelial basal lamina, spread through the underlying muscle and finally metastasis to lymph nodes, liver, lungs and other tissues is a likely outcome in later stages of colorectal cancer (see Figure 19.25).

Whilst there are many possible paths in the progression of colorectal cancer, there are some trends that emerge. Mutations in *APC*, *p53*, and *K-ras* are extremely common (each occurring in about 80%, 60% and 40% of colorectal cancers respectively), and there are some other genes (*Smad4*, *TGFrII*, β -catenin and DNA mismatch repair genes) that are mutated in a lower percentage of cases. In 26% of cases, especially in metastatic colon cancers, there is altered expression of a family of tyrosine phosphatases, which are thought to act normally as tumour suppressors by controlling the activity of kinase cascades. It is likely that there are a minimum number of critical mutations that need to be acquired in key genes, (triggering each of the hallmarks of cancer described above) for the cancer to become fully malignant. Moreover, it is becoming apparent that some of the critical mutations are usually acquired earlier in tumour progression than others (see Figure 19.26).

The *APC* gene has been found to be deleted in individuals with familial adenomatous polyposis (FAP). These individuals develop hundreds of colonic polyps at an early age and, if not removed, one or more of these polyps will almost invariably progress to cancer within a decade or two. Even in non-familial cases of colorectal cancer, *APC* inactivation is frequently a very early step. Loss of *APC* appears to increase cell proliferation, without changing the histology of the cells significantly. *APC* is an inhibitory component in the Wnt signalling pathway (shown in very simplified form in Figure 19.24 and in more detail in Figure 17.15). *APC* promotes degradation of β -catenin, which would otherwise help to activate transcription of *myc* and other Wnt target genes. So *APC* is a tumour suppressor gene and β -catenin is acting as a proto-oncogene. The Wnt pathway is particularly important in maintaining the gut epithelial stem cell population (Section 17.4.1), which helps to explain why at least two different components of the pathway are implicated in the development of colon cancer.

Increased genetic instability can also be a precipitating event in colorectal cancer. This is most apparent in patients with HNPCC (Section 19.2.1), who have inherited a mutation in one of the DNA mismatch repair genes (usually *hMLH1* or *hMSH2*).

**Figure 19.25**

Cross-sections and diagrammatic interpretations showing the stages in development of a typical colon cancer. (a) An adenomatous polyp from the colon. The polyp protrudes into the lumen – the space inside the colon. The rest of the wall of the colon is covered with normal colonic epithelium; the epithelium on the polyp appears mildly abnormal. (b) A carcinoma that is beginning to invade the underlying muscle layer.

These patients do not have the profusion of early hyperplastic polyps found in FAP patients (hence 'non-polyposis'), and their gross karyotype looks fairly normal.

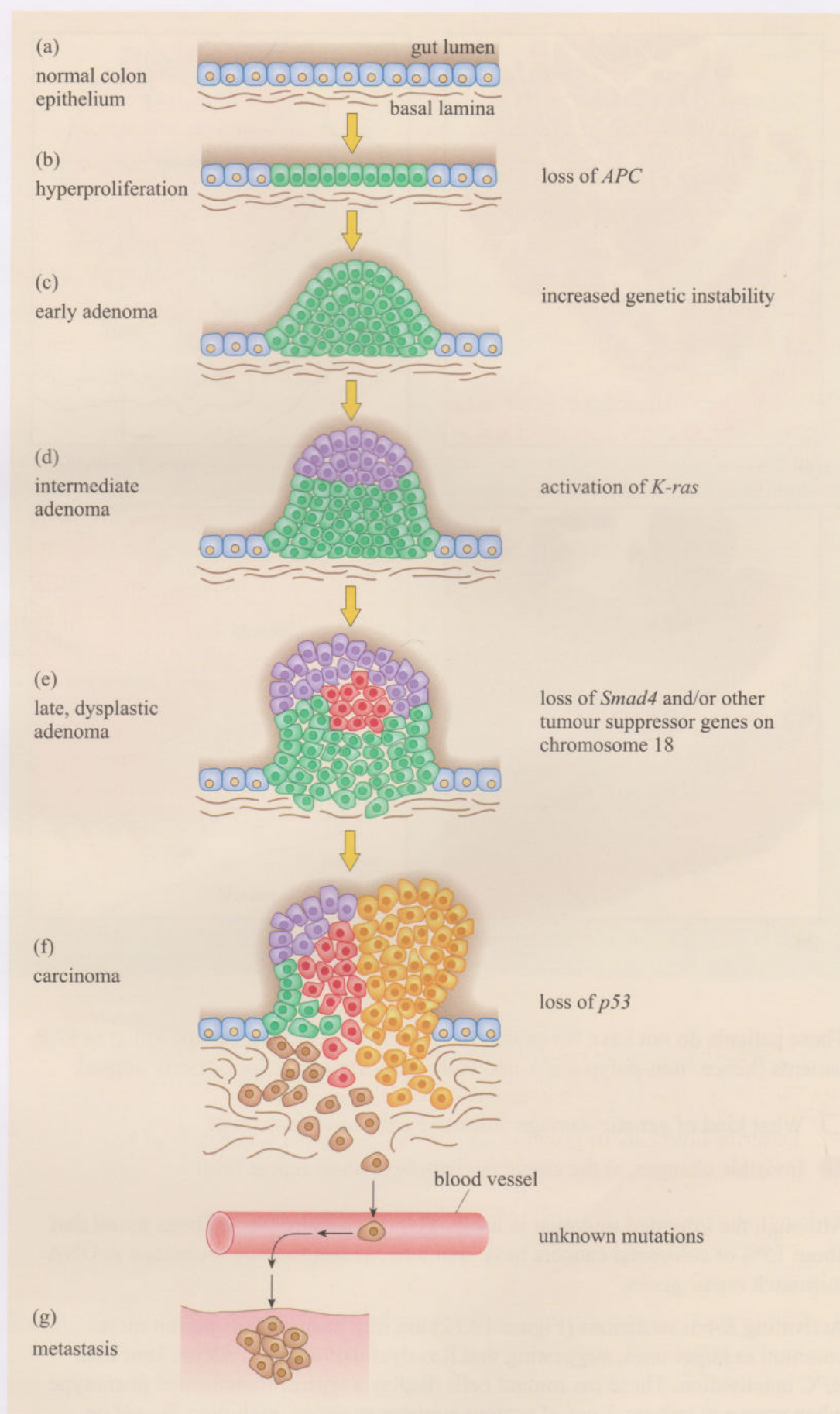
- ☐ What kind of genetic damage will these cells have sustained?
- ☒ Invisible changes, at the single nucleotide or short repeat level.

Although the inherited mutation is fairly rare, it has subsequently been found that about 15% of colorectal cancers have spontaneous inactivating mutations in DNA mismatch repair genes.

Activating *K-ras* mutations (Figure 19.12) are rare in small polyps, but more common in larger ones, suggesting that Ras dysfunction often occurs later than *APC* inactivation. These *ras* mutant cells display a typical transformed phenotype when grown in culture. Loss of tumour suppressor genes, including *Smad4* on chromosome 18 from which segments are often lost, is more commonly seen later still in the progression of colorectal cancer.

Figure 19.26

A generalized diagram of the possible cellular and molecular progression of colorectal cancer. Many other sequences of genetic lesions are likely too. Increased proliferation of colon epithelium is characterized by the loss of *APC* function. Adenomas are characterized initially by increased genetic instability and later by activation of *K-ras* by point mutation and loss of *Smad4* and/or other tumour suppressor genes on chromosome 18. Colon carcinoma also presents loss of *p53*, whereas the mutations that induce metastasis are unknown.



- What is the normal role of Smad4?
- It transduces the signal from TGF receptors (as discussed in Section 19.4.1).

TGFrII, a gene encoding the TGF β receptor type II, is also found to be mutated in 10% of colorectal cancers. We have seen earlier that the TGF β pathway prevents cyclin D from inactivating Rb by phosphorylation, so Smad4 and TGFrII are normally acting as tumour suppressors and are inactivated in colorectal cancer.

Of course, these are only the mutations that have been identified and many more will exist in genes that are yet to be examined. Moreover, each tumour undergoes its own progressive accumulation of mutations and ends up not as one tumour, but as a heterogeneous collection of tumour genotypes. However, it is not always a *totally* random process, as we have seen that some mutations, such as *p53* deletion, predispose to certain types of further genetic damage, and that certain tumours are more likely to contain lesions in genes involved in the differentiation of that particular cell type. This opens some windows of opportunity for the development of gene-specific therapies, as discussed in Box 19.6.

Summary of Section 19.9

- 1 Colorectal cancer is a suitable model for the study of tumour progression, due to the availability of surgically removed early polyps. There are characteristic genetic lesions, some relating to the specific cell type, and some tending to occur earlier or later in the progression.
- 2 Mutations in the Wnt pathway genes (*APC* and β -*catenin*), which maintain gut epithelial stem cells, are common in human colorectal cancer; *APC* gene mutations are found in patients with familial adenomatous polyposis coli.
- 3 Mutations of *p53* and *K-ras* are particularly common in human colorectal cancer, while mutations in genes encoding proteins of the TGF pathway (*Smad4*, *TGFrII*), in DNA mismatch repair genes and in tyrosine phosphatase genes are found at lower frequencies.

Learning outcomes for Chapter 19

When you have studied this chapter, you should be able to:

- 19.1 Define and use each of the terms printed in **bold** in the text.
- 19.2 Describe how tumour growth requires the successive acquisition of key properties derived from cumulative mutations.
- 19.3 Explain how genetic instability arises and how it contributes to tumour progression.
- 19.4 Explain how gain-of-function mutations arise, give examples of oncogenes and describe their mode of action.
- 19.5 Explain how loss-of-function mutations arise, give examples of tumour suppressor genes and describe their mode of action.
- 19.6 Discuss apoptosis in its role as a cellular response that controls abnormal cell proliferation.

- 19.7 Describe the causes and consequences of telomere loss and telomerase reactivation
- 19.8 Apply basic concepts in cell migration to tumour growth, specifically in relation to angiogenesis and tumour invasion and metastasis.
- 19.9 Relate current and novel therapeutic approaches to knowledge of cancer characteristics, with examples.

Questions for Chapter 19

Question 19.1

What is meant by a 'microevolutionary process' in relation to tumorigenesis?

Question 19.2

Give an example of a gene that when mutated gives rise to genetic instability, and explain how it operates.

Question 19.3

Explain why tumour suppressor genes cannot be detected by classical transformation assays.

Question 19.4

Name one oncogene and one tumour suppressor gene associated with evading apoptosis.

Question 19.5

How might a cell escape replicative senescence due to telomere erosion?

Question 19.6

Describe the role of VEGF in tumorigenesis.

Question 19.7

Give an example of a tailored therapeutic approach that uses antibody technology.

References

- van't Veer, L. J., Dai, H. van de, Vijver, M. J., He, Y. D., Hart, A. A. M., Mao, M., Peterse, H. L., Van der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R. and Friend, S. H. (2002) Gene expression profiling predicts clinical outcome of breast cancer, *Nature*, **415**, pp. 530–536.
- Fearon, E. R. and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis, *Cell*, **61**, pp. 759–767.

Paez, J. G., Jänne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M. J., Sellers, W. R., Johnson, B. E. and Meyerson, M. (2004) *EGFR* mutations in lung cancer: correlation with clinical response to Gefitinib therapy, *Science*, **304**, pp. 1497–1500.

Further sources

Abbott, A. (2002) Cancer research: on the offensive, *Nature*, **416**, pp.470–474.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002) *The Molecular Biology of the Cell* (4th edn), Garland Science, New York.

Berg, J.M., Tymoczko, J. L. and Stryer, L. (2001) *Biochemistry* (5th edn), W. H. Freeman and Company, New York.

Cahill, D. P., Kinzler, K. W., Vogelstein, B. and Lengauer, C. (1999) Genetic instability and Darwinian selection in tumours, *Trends in Cell Biology*, **9**(12), pp. M57–M59.

Coussens, L. M and Werb, Z. (2002) Inflammation and cancer, *Nature*, **420**, pp. 860–867.

Evan, G. I. and Vousden, K. H. (2001) Proliferation, cell cycle and apoptosis in cancer, *Nature*, **411**, pp. 342–348.

Folkman, J. (2002) Looking for a good endothelial address, *Cancer Cell*, **1**, pp. 113–115.

Hanahan, D. and Weinberg, R. A. (2000) The hallmarks of cancer, *Cell*, **100**, pp. 57–70.

Hurley, L. H. (2002) DNA and its associated processes as targets for cancer therapy, *Nature Reviews Cancer*, **2**, pp. 189–200.

Jones, P. A. and Baylin, S. B. (2002) The fundamental role of epigenetic events in cancer, *Nature Reviews Cancer*, **3**, pp. 415–428.

Knudson, A. G. (2001) Two genetic hits (more or less) to cancer, *Nature Reviews Cancer*, **1**, pp. 157–162.

Lakhani, S. R. and Ashworth, A. (2001) Microarray and histopathological analysis of tumours: the future and the past?, *Nature Reviews Cancer*, **1**, pp. 151–157.

Saffrey, J. (ed.) (2001) S204 Book 3 *The Core of Life* Vol. II, Ch. 10, The Open University, Milton Keynes.

Shawver, L. K. Slamon, D. and Ullrich, A. (2002). Smart drugs: tyrosine kinase inhibitors in cancer therapy, *Cancer Cell*, **1**, pp. 117–123.

Tannock, I. F. and Hill, R. P. (1998) *The Basic Science of Oncology* (3rd edn), McGraw-Hill, Toronto.

Watson, J. D., Hopkins, N. H., Roberts, J. W., Steitz, J. A. and Weiner, A. M. (1987) *Molecular Biology of the Gene* (4th edn), The Benjamin/Cummings Publishing Company, Menlo Park.

EPILOGUE

At the beginning of this course, we looked at some of the cell structures that characterize the cells of present-day organisms, and consider how these emerged from primordial cells. It is quite astounding that life in all its complexity could have developed from sets of interacting prebiotic molecules, obeying the basic laws of physics and chemistry. In this course, we too have followed a route starting with the biological chemistry of molecules such as proteins and DNA, then on to the diversity of cellular processes that maintain the function of cells, and ending with the complexities of cell–cell interactions as illustrated by those occurring during development. One of the most exciting aspects of modern cell biology is that we can start to understand complex physiological processes at both a cellular and a molecular level.

Our choice of tumorigenesis as the final chapter was quite deliberate. Not only has this subject been a key driver of research in cell biology, it also illustrates how an integrated knowledge of molecular and cell biology can help us to understand important physiological and pathological conditions in mammals. The development of treatments and diagnostic tests for human cancer will require knowledge of the stability of DNA and its repair, an understanding of the cell cycle and its control and an insight into the processes of cell proliferation, death and motility. In each case, understanding how tumours may develop requires a clear knowledge of how cells of different types function normally.

There has been an enormous expansion of knowledge in cell biology over the last 20 years. We have tried to present the basic principles of the subject, while providing sufficient examples for you to appreciate the range and depth of knowledge that is available and where the boundaries of our knowledge lie. We also hope that you have gained the skills that will allow you to read further in the scientific literature, and to follow how different techniques are used to analyse cell functions.

Most of all, we hope you have enjoyed the course.

ANSWERS TO QUESTIONS

Question 16.1

Macrophages, neutrophils, lymphocytes, endothelium, fibroblasts, osteoclasts, skin epithelium (keratinocytes). Migration of leukocytes is essential for immune defence whereas the other cell types may move to participate in tissue repair or remodelling.

Question 16.2

E-cadherin-transfected fibroblasts adhere to each other: cadherins mediate homologous adhesion via homophilic interactions (that is, between molecules of the same kind); ICAM-1 binds in a heterophilic way to leukocyte integrins LFA-1 and CR3. One would therefore not expect transfection of fibroblasts with ICAM-1 to affect the interaction of fibroblasts with other fibroblasts (unless they had been previously transfected with leukocyte LFA-1 or CR3).

Question 16.3

C5a is a chemotactic peptide that attracts phagocytes to sites of inflammation.

Cdc42, a small GTPase that controls global reorganization of the F-actin network.

CXCR1 is a 7TM G protein-coupled chemokine receptor, which allows cells to respond to some chemokines, for example IL-8.

Ezrin is an anchoring protein that cross-links certain transmembrane proteins such as the Ig superfamily cell adhesion molecules (e.g. ICAM-1) to actin filaments.

PI 3-kinase is a lipid-modifying kinase that phosphorylates membrane phospholipids, specifically phosphatidylinositols at position 3, generating docking sites for PH domain-containing proteins required for adhesion/migration at the leading edge of a migrating cell.

Robo-1 is a receptor that binds Slits, which are chemotaxis inhibitory signals.

Vinculin is an anchoring protein that links integrins to the actin filament network.

Question 17.1

Asymmetric division is the unequal division of a cell, such that the two daughter cells are different in their contents, and possibly also in their size. In early *C. elegans* development, the first division of the zygote is asymmetric, and results in the formation of the anterior AB cell and the smaller, posterior P₁ cell. Further asymmetric divisions occur, and serve to specify the subsequent development of lineages of cells that give rise to different cell types in the adult. In the intestinal epithelium, stem cells near the bases of the crypts divide asymmetrically, giving rise to one daughter that is a stem cell, and one that will, in normal circumstances, go on to become first a proliferating potential clonogenic cell, then a transit cell and finally differentiate into an intestinal epithelial cell.

Question 17.2

Contact-mediated cell–cell interactions via the cell surface ligand Delta and its receptor Notch triggers the differentiation of neurons. Initially, all the cells of the neuroectoderm are equivalent, and express Notch and Delta. Upregulation of expression of Delta by one cell causes increased signalling via Notch in its neighbours. Since Notch signalling inhibits expression of the proneural gene neurogenin, neuronal differentiation is inhibited in these cells, while it is promoted in the first cell, because the increased expression of neurogenin promotes expression of NeuroD, and downstream neural genes.

Question 17.3

The presumptive endoderm and mesoderm stream inwards to the middle of the embryo, from the blastopore. The process results in the formation of the three germ layers and the specification of the neural plate and the A–P axis.

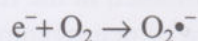
Question 17.4

Migrating neural crest cells do so along specific routes in the embryo; for example, within the anterior but not the posterior portion of the somites. This specificity is thought to be due to repulsion, as the migrating crest cells express Eph receptors, while the cells of the posterior somites express ephrin.

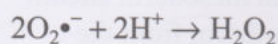
The trajectory of the axons of commissural neurons in the developing spinal cord depends upon diffusible and contact-mediated repulsive signals. The repulsive protein Slit is expressed by the ventral midline cells. Once the growing axons cross the ventral midline, they upregulate the Slit receptor, Robo, so are repelled from crossing back across the midline. The developing spinal cord itself expresses the diffusible repulsive protein, semaphorin, so the growing axons are also repelled from this region, and therefore grow between the two repulsive stimuli, towards the brain.

Question 18.1

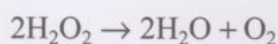
Superoxide ($O_2^{\bullet-}$) possesses an unpaired electron, and so is a free radical; hydrogen peroxide (H_2O_2) is not a free radical because it does not possess an unpaired electron. Both superoxide and hydrogen peroxide, however, are oxidants, and known as reactive oxygen species (ROS). Both are generated in mitochondria. Superoxide is produced by partial reduction of O_2 by electrons that have leaked through the inner mitochondrial membrane:



Hydrogen peroxide is produced as a result of the action of the mitochondrial antioxidant enzyme MnSOD, on superoxide radicals, which thus protects against superoxide damage:



Another antioxidant enzyme, catalase, converts hydrogen peroxide to water and oxygen in both the mitochondrial matrix and the cytosol:



Question 18.2

Free radicals cause double-strand breaks and also cause oxidative damage, resulting in the formation of altered bases and nucleosides such as 8-oxoguanosine. In

replicative cells, this DNA damage can trigger apoptosis or replicative senescence, i.e. irreversible withdrawal from the cell cycle, possibly by an effect on the telomeres. In post-mitotic cells, damage to mitochondrial DNA can cause mutation of some mitochondrial genes, e.g. those that encode components of the ETC, such as subunit I of the cytochrome oxidase. Such mutations affect the efficiency of cellular respiration in that mitochondrion.

Question 18.3

DAF-16 is a transcription factor in *C. elegans*. It acts to promote expression of some antioxidant enzymes and stress response proteins. A mutant DAF-16 with increased activity would be expected to increase expression of these proteins and extend lifespan in *C. elegans*.

Question 19.1

The acquisition of mutations that gives the tumour cell and its progeny a 'competitive advantage' over surrounding cells, for example by increasing proliferation or survival.

Question 19.2

Any one of the following:

MLH/MSH, which are genes for mismatch repair proteins (associated with hereditary non-polyposis colorectal cancer, HNPCC).

XPA-G, which are genes involved in nucleotide excision repair (associated with xeroderma pigmentosum, XP).

BRCA1 and 2, which are genes involved in various repair processes, including nucleotide excision repair and homologous recombination (associated with breast and ovarian cancer).

ATM, whose product detects double-strand breaks, leading to cell cycle arrest (associated with ataxia telangiectasia, AT).

p53, raised in response to DNA damaging events, leading to blocking progression through S phase of the cell cycle, and giving the cell time to check the status of its DNA before proceeding with DNA synthesis (mutated in half of all human cancers).

Question 19.3

They cannot be detected by transformation assays because any effect of the introduced loss-of-function version would be obliterated by the recipient cell's normal versions of the gene.

Question 19.4

Bcl-2 is an oncogene, and *p53* and *PTEN* are tumour suppressor genes.

Question 19.5

A cell could escape replicative senescence caused by telomere erosion if it had sustained *p53*-inactivating mutations, or if it had switched on telomerase activity.

Question 19.6

VEGF is synthesized by normal cells in response to hypoxia, and also by tumour cells. VEGF induces endothelial cells to migrate toward the VEGF signal, proliferating and forming new capillaries.

Question 19.7

You may have thought of Herceptin, which is a monoclonal antibody that blocks HER2 receptors on breast epithelial cells and is used in the treatment of human breast cancer.

ACKNOWLEDGEMENTS

Every effort has been made to contact copyright holders. If any have been inadvertently overlooked, the publishers will be pleased to make the necessary arrangements at the first opportunity. Grateful acknowledgement is made to the following sources for permission to reproduce material within this book.

Cover image: Science Photo Library. This image was also used on Book 1 and Book 2 of S377 and should have been acknowledged in each of these books. We apologise unreservedly for these omissions and promise to rectify them at the earliest opportunity.

Chapter 15

Figure 15.1 Pace, N., Meyer, L. B. and Vaughan, B. E. (1956) *Journal of Applied Physiology*, **9**, The American Physiological Society; *Figure 15.2* Reprinted from *Immunology: An Illustrated Outline*, 4th edn (2004), Figure 1.11 from the article 'Leucocyte development' by Male, D., copyright © 2004, with permission of Elsevier.

Chapter 16

Figure 16.1 From Alberts, B. *et al.* (2002) *Molecular Biology of the Cell*, 4th edn, Garland Science, reproduced with permission of Routledge/Taylor and Francis Books, Inc.; *Figure 16.2* Male, D. (1998) 'Steps in leucocyte migration into tissues', *Immunology: An Illustrated Outline*, 3rd edn, Mosby International Ltd; *Figure 16.6a, b* Reprinted from *Clinical Immunobiology*, Brostoff, J. *et al.*, p. 144, copyright © 1991 with permission from Elsevier; *Figure 16.14b* Courtesy of E. Solito, Department of Neuroendocrinology, Imperial College, London; *Figure 16.5* Courtesy of C. Lancashire, Department of Biological Sciences, Open University, Milton Keynes; *Figure 16.17a, b* Reproduced from *The Journal of Cell Biology*, 1999, **145** (5), p. 1020, by copyright permission of The Rockefeller University Press; *Figure 16.20* Copyright © 1998, From Chapters 16 and 17, *Essential Cell Biology: An Introduction to the Molecular Biology of The Cell*, by Alberts, B. *et al.*, reproduced by permission of Taylor and Francis Books, Inc./Routledge Inc., <http://www.routledge-ny.com>; *Figure 16.23* Reprinted with permission from A. Hall, *Science*, **279**, pp. 509–514, 1998, copyright © American Association for the Advancement of Science.

Chapter 17

Figure 17.1 Courtesy of I. Romero, Department of Biological Sciences, Open University, Milton Keynes; *Figure 17.3* Michael Whitaker/Science Photo Library; *Figures 17.8, 17.11* After K. Kemphues, *Cell*, 2000, **101**, pp. 345–348; *Figure 17.9* Wodarz (2002) 'Establishing cell polarity in development', *Nature Cell Biology*, **4**, February 2002, Nature Publishing Group; *Figure 17.21b* Courtesy of J. Golding, MRC Clinical Sciences, Hammersmith, London; *Figure 17.22* Based on a diagram by William McGinnis; *Figure 17.23* Figure 4.12 from 'Hox gene expression along the antero-posterior axis of the mouse mesoderm', in *Principles of Development*, by Wolpert, L. *et al.* (2001), by permission of Oxford University Press; *Figure 17.25b* Reprinted from Borday, C. *et al.*, 'Developmental molecular switches regulating breathing patterns in CNS', *Respiratory Physiology and Neurobiology*, **135**, 2003, p.123, copyright © 2003, with permission from Elsevier; *Figure 17.26* Jessell, T. M. (2000) 'Neuronal specification in the spinal cord:

inductive signals and transcriptional codes', *Nature Reviews Genetics*, **1**, October 2000, Nature Publishing Group; *Figure 17.29* Beddington, R. *et al.* (2003) *Principles of Development*, 2nd edn, Oxford University Press; *Figure 17.31* Goldberg, D. J. and Burmeister, D. W. (1989) *Trends in Neurosciences*, **12**, Elsevier Science Publishers; *Figure 17.35* Potten, C. S. *et al.* (2003) 'The small intestine as a model for evaluating adult tissue stem cell drug targets', *Cell Proliferation*, **36**, 2003, copyright © 2003 Blackwell Publishing Limited.

Chapter 18

Figure 18.2 Courtesy of Tim Cowen, Department of Anatomy and Developmental Biology, University College, London; *Figures 18.3, 18.4* Courtesy of I. Kill, Department of Biological Sciences, Brunel University, Uxbridge; *Figure 18.8* Taylor, R. W. (2003) 'Mitochondrial DNA mutations in human colonic crypt stem cells', *Journal of Clinical Investigation*, **112**, November 2003, reproduced with permission of the *Journal of Clinical Investigation*, via Copyright Clearance Center; *Figure 18.9* Adapted from Longo, V. D. and Finch, C. E. (2003) 'Evolutionary medicine: from dwarf model systems to healthy centenarians?', *Science*, 28 February 2003, American Association for the Advancement of Science.

Chapter 19

Figures 19.1, 19.2 Tannock, I. F. and Hill, R. P. (1998) *The Basic Science of Oncology*, by permission of McGraw-Hill Companies, Inc; *Figure 19.4* Courtesy of Wael Abdel-Rahman and Paul Edwards; *Figures 19.5, 19.13, 19.14, 19.15, 19.17, 19.18, 19.19, 19.23* From Alberts, B. *et al.* (2002) *Molecular Biology of the Cell*, 4th edn, Garland Science, reproduced with permission of Routledge/Taylor and Francis Books, Inc.; *Figure 19.8 (a) top left, (b) top right* Reprinted from 'Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and transformation of NIH 3T3 cells', by Cowley, S. *et al.*, *Cell*, **77**, copyright © 1994 by Cell Press, with permission from Elsevier; *Figure 19.9* Lakhani, S. R. and Ashworth, A. (2001) 'Microarray and histopathological analysis of tumours: the future and the past?', *Nature Reviews Cancer*, **1**, November 2001, copyright © Nature Publishing Group; *Figure 19.10* van't Veer, L. J. *et al.* (2002) 'Gene expression profiling predicts clinical outcome of breast cancer', *Nature*, **415**, 31 January 2002, copyright © Nature Publishing Group; *Figure 19.16* Reprinted with permission from J. J. Yunis (1983) *Science*, **221**, copyright © American Association for the Advancement of Science; *Figure 19.21* Cahill, D. P., Kinzler, K. W., Vogelstein, B. and Lengauer, C. (1999) 'Genetic instability and Darwinian selection in tumours', *Trends in Cell Biology*, **9** (12), 1999, Elsevier Science Ltd; *Figure 19.24* Reprinted from Hanahan, D. and Weinberg, R. A. (2000) 'The hallmarks of cancer', *Cell*, **100**, January 2000, copyright © 2000 by Cell Press, with permission from Elsevier; *Figure 19.25* Courtesy of Paul Edwards, Department of Pathology, University of Cambridge.

INDEX

Note: Entries in **bold** are key terms. Page numbers referring to information that is given only in a figure or caption or a table are printed in *italics*.

A

AB cells 61, 62, 64
abl gene 153–4
 ablation, cell 59
 absorptive enterocytes 90
 α -actinin 35
 actin
 anchoring proteins 34–6, 43
 cadherin interaction 16
 in cell migration 11, 12
 polymerization 32–4
activin 67–8
ADAMs (metalloprotease disintegrins) 46
 adenomas 134, 176, 177
 adherens junctions 14, 15
 adhesion 8–9, 13, 14, 42–5, 171
 anchoring proteins 34–6
 cell-surface expression 27–9
 immunoglobulins 22–3
 selectins and sialoadhesins 25–6
 surface grouping 29–31
 see also cadherins; integrins
 adrenomedullary cells 82, 85, 95
 adult stem cells 50, 93–4, 111, 120, 121
affinity, binding 13, 18
 aflatoxin, cancer risk factor 128
 AGEs (advanced glycosylation end-products) 122
 ageing 99–100
 cancer risk factor 128, 129
 and free radicals 101–8
 genomic instability 123
 insulin signalling pathway 124–5
 mitotically active cells 108–19
 post-mitotic cells 119–22
 see also adult cells; senescent cells
 alkylating agents 140, 141
 allosteric changes, integrins 20–1
 alternative splicing, of exons 24
 Alzheimer's disease 122
 amino acids, oxidative damage 104–5

amino acid radicals 105
amyloidoses 122
anchorage dependence 23
 anchoring junctions 7
 anchoring proteins 34–6
angiogenesis 168–70
 animal cap cells 66, 67
 animal development, overview 51–6
 animal pole 52, 55, 60, 70
 Antennapedia complex 77, 78
 anterior–posterior (A–P) axis 52, 55, 57, 63
 central nervous system 76–7, 79
 antibiotics, use in cancer treatment 141
 antimetabolites 140, 141
 antioxidant defences 105, 106, 107
 antisense oligonucleotides 151, 167
APC mutations 68, 174, 176
Aplysia (sea-slug), growth cone 87
 apoptosis 4, 21, 117, 173
 evasion 135, 162–4
 p53 activation 114–15
 archenteron 55, 57
ARP complex 33, 45
 ascorbic acid *see* vitamin C
 astrocytes 49
 see also glial cells
asymmetric division 61–2, 63
 ataxia telangiectasia 100, 123, 138
ATM 114, 115, 117, 138
ATR 129
 autocrine stimulation 150
avidity, binding 13, 28, 29
axon guidance 73, 86–8
 growth cone 86, 87
 azidothymine (AZT) 167

B

B cells
 tumorigenesis 156, 157
 differentiation 3

basal lamina 7, 24, 25, 133, 172, 174, 176
 Bcl-2 protein 163, 164, 173
 Bcr-Abl protein 154, 155
bcr gene 154
 benzo(a)pyrene 128
 bicoid protein 65
 Bithorax complex 77, 78
 blastocoel 52, 54, 57
 blastocytes, culture 94
blastopore 52, 55, 56, 73
blastula 52, 54, 55
 mesoderm induction 66, 68
 blood clotting 28
 BMPs 58, 69, 70, 81
 in neural induction 73–4
 bone morphogenetic proteins *see* BMPs
 brain development 76, 80
BRCA genes 128, 130, 137–8
 BrdU DNA labelling 92, 109
 breast cancer
 cadherin function 16
 cell replication 165, 169
 genetic predisposition 128, 137
 microarray analysis 148
 oncogenes 150, 151, 163
 Burkitt's lymphoma 143, 156, 157

C

C5a polypeptide 38, 40
cadherins 9, 14–16
 E-cadherin 14, 16, 84, 171, 173
 VE-cadherin 14
Caenorhabditis elegans (nematode worm)
 ageing 99, 108
 cadherins 14–15
 early development 61, 62, 63, 64
 insulin signalling pathway 124–5
 calcium ions
 cell adhesion 15, 18
 egg fertilization, calcium wave 53–4
 cAMP 32, 37
 camptothecins 141
 cancer 128, 134
 DNA damage detection 138–42
 DNA repair 136–7
 molecular pathways 173

therapy 140–1, 151, 161, 167, 169
 tumour progression 174–7
see also breast cancer; colorectal cancer; leukaemia;
 lung cancer

cancer-critical genes 131

identification 146–8

capping proteins 32, 33

carbon-centred radicals 101, 102, 104, 106

carbonyl groups 105

carcinogens 128, 129

carcinomas 134, 175, 176

caspases 163, 173

catalase 103, 106, 124

catenins 14, 16

β -catenin 68–9, 70, 173

Cdc42 protein 42–3, 62

cDNA microarrays 148, 172

cell adhesion molecules (CAMs) 8

 cadherins 9, 14–16, 171, 173

 cell surface expression 9, 27–9

 cell surface grouping 29–30

 Ig superfamily 14, 22–3, 26

 integrins 14, 17–19, 20–1, 22–5, 29–30, 35, 44, 171, 173

 selectins 10, 14, 25–6, 29

 sialoadhesins 26

cell–cell adhesion 14

cell–cell signalling proteins 56, 58

cell death *see* apoptosis

cell–matrix adhesion 14

cell migration 7–8, 9–11, 12

cell transfection 147

cells

 ablation 59

 ageing 99–100

 culture 60

 differentiation 89–96

 division 2–4, 108–9

 functions 5–6

 interactions 30–1, 64

 junctions 7, 14

 migration 7–8, 9–11, 12

 mechanisms 32–6

 of neural crest cells 82–5

 polarization 42–3, 62–3

 proliferation 131, 132, 133, 173

 tracing 59

see also endothelial cells; epithelial cells; stem cells

cervical cancer 160
 chaperones 122
 chemoattraction 86, 87
chemokines 38–9, 40
 chemokine receptors 39–40
chemokinesis 11
 chemorepulsion 86, 87
 chemotactic molecules 37–9
chemotaxis 11
 control of 42–5
 mechanisms 32–6
 chemotherapy 140–1
 chick embryo 9
 CNS 76
 Hox genes 80
 motor neurons 82
 Chordin 73
 chromosomal damage 130–1, 139, 164–7
 chromosome painting 137
 chronic myelogenous leukaemia (CML) 153, 154, 155
cleavage of embryo 52, 54, 55
 clonal extinction 133
 clones, tumour origin 132, 133, 134
 cloning 95, 96
 coding region of DNA 130
cofilin 33, 34
collagen 18, 24
 colorectal cancer
 cell replication 152, 163, 165
 FAP 128, 175
 HNPCC 136
 risk factors 128, 136
 tumour progression 174–7
combinatorial control 65, 66–71
commissural neurons 86, 88
committed cell 50
 communicating junctions 7
 comparative genome hybridization (CGH) 146, 147
 constitutive
 protein synthesis 28
 activation 92, 142, 150, 153
 contact attraction 86, 87
 contact repulsion 86, 87
 cortex proteins, and asymmetric division 62, 63
 CR3 18, 19
 CR4 18

crypts 90–1, 120, 121
 cultured cells 60, 94
 co-culture 66, 67
 oncogene identification 146, 147
 senescence studies 109, 110, 111, 113, 114, 116, 118
 CXC chemokine ligands 38–9
 cyclins, in senescent cells 112–13
 dysregulated in cancer 156, 159, 173
 cytochrome oxidase 102, 103, 120, 121
 cytokines 3, 4
 cytoskeleton
 anchoring proteins 16
 integrin binding 18
 organization 43, 44
 cytosolic domains, integrin role in activation 20–1
 cytosolic regulator of adenylyl cyclase (CRAC) 41, 44
cytotoxic therapies 140–1

D

DAF-16 124, 125
 decoy receptors 163, 173
 de-differentiation 95
 Delta proteins 58, 64, 74–5
 desmocollin 14
 desmoglein 14, 16, 17
 desmosomes 14, 15, 16
determined cell 50
development 50
 nervous system 71–89
 overview of 51–6
 Dickkopf 58, 92
Dityostelium discoideum (slime mould)
 cell migration 10, 32, 34
 chemotaxis 37
 myosin II 36
 signalling systems 40, 41, 42, 44
differentiation 49–51
 animal development 51–6
 common mechanisms 61–6
 endogenous inhibitors 58
 mature tissues 89–96
 methods of study 59–60
 molecules involved 56, 57, 58
 nervous system 71–89
Dishevelled (Dsh) 58, 68–9
 disintegrins 46

DNA

- amplification 156
- assays in oncogenes 146, 147
- replication in intestinal stem cells 91–2
- methylation 159
- repair 128, 136–7
- telomeric 114, 116–18
- tumour viruses 160–1

DNA damage

- cancer therapies 140–1
- detection mechanisms 138–42, 173
- genomic instability 123
 - see also* genetic instability
- mitochondrial 119–20, 121
- mutations 127–32, 133
- oxidative 104

dorsal root ganglia 85

dorso-ventral (D–V) axis 54, 57

- protein signal gradients 67, 69, 70, 73
- spinal cord 81, 82

Drosophila melanogaster

- ageing 99
- cell differentiation 63, 65
- chemotaxis inhibition 45
- Hox genes 77, 78
- insulin signalling pathway 124
- regional segmentation 77
- Robo receptors 45

dysplasia 134, 135, 176**E**

E-cadherin 16, 171, 173

E cell 61, 62, 64

E6 viral protein 160–1, 173

E7 viral protein 160–1, 173

E-selectin 16, 25–6, 29

ECM *see* extracellular matrix**ectoderm** 8, 54, 55

- neuronal induction 72, 73

EGF receptor family 150, 154

egg

- differentiation 62, 65
- fertilization 52, 53–4

eicosanoids 38

electron transport chain (ETC) 101–2, 119–20

embryo

- cell migration 9

cloning 95–6

development 55–6, 57

differentiation 59–66

neural crest cells 82–5

embryonic stem (ES) cells 94

EMS cells 61, 62, 64

endoderm 8, 54, 55, 67

endostatin 168, 170

endothelial cells 10, 49

- leukocyte adhesion 10–11, 28, 29

- tumours 168–70

enteroendocrine cells 90, 91

environmental carcinogens 128

Eph receptors 58, 85

ephrins 58, 77, 85

epithelial cells 63

- in cancer 134

- intestinal 90–3

- see also* carcinomas

epithelial–mesenchymal transition 84

epithelium 90

Epstein-Barr virus (EBV) 156

ERM family of proteins 34–5

erythrocytes 2

erythropoietin 2–3

expression microarrays *see* cDNA microarrays

extracellular ligands 14, 17

extracellular matrix (ECM) 4, 23–5, 29–30, 65, 85, 168, 169

- cell migration 11, 12

- proteins 14, 23–5

- tumour cell migration 171

ezrin 34, 35**F**

F-actin 33, 34, 43, 45

- see also* actin

familial adenomatous polyposis coli (FAP) 128, 174, 175

Fas pathway 163, 173

fate map 59, 66

fertilization, egg 52, 53–4, 62

FGF family of proteins 58

FGFR3 receptor 150

fibroblasts

- interactions with other cells 169

- senescence studies 109–11, 113, 116

- transformation *in vitro* 146, 147, 164

fibronectin 18, 24, 25
 fibrosarcoma 149
filopodia 10, 11, 43
 axon guidance 86, 87
 fluorescence *in situ* hybridization (FISH) 146, 147, 156
focal adhesions 14, 17, 29, 44
focal adhesion kinase (FAK) 30, 31
 Forkhead transcription factor 124
 formylmethionine (fMet) 38
 Fos 173
 founder cells 61, 62
free radicals 101–3, 136
 cellular defences 105, 106
 reactions with molecules and cells 103–5
 studying 107
‘free radical theory’ 100
 Frizzled family of receptors 64, 68, 69, 173
 frog *see Xenopus laevis*
 fruit-fly *see Drosophila melanogaster*
 ‘futile cycles’ 107
 Frzb 58, 73

G

G protein-coupled receptors (GPCR) 37, 40–1
 G-quadruplex ligands 167
 gain-of-function mutations 142–8
 gap junctions 7, 14
gastrulation 52, 55–6
 neural induction 73–4
 GATA-1 transcription factor 74
 Gefinitib 154–5
 genes
 ageing process 100, 124–5
 cancer-critical 146, 147, 148
 DNA repair 136–7
 mutations 131–2
 tumour suppressors 157–61
 see also Hox genes; oncogenes
 gene amplification 143, 144
 gene expression
 differential 50–1, 59
 microarrays 148
 senescent cells 112–13
 gene silencing 158, 159
 gene therapy 161

genetic instability
 ageing 123
 colorectal cancer 174–5, 176
 senescence 114, 117
 tumorigenesis 123, 132, 135–42, 158, 166
 genetic predisposition *see* inherited mutations
 genomic analysis 60, 146, 147, 148
 genomic instability *see* genetic instability
germ layers 54, 55
 germ (cell) line 61, 62
 Gleevec 154, 155
 glial cells 49, 71, 72, 74, 75, 85, 93
 tumour (glioma) 150
 see also astrocytes
 glutathione (GSH) 106
 glutathione peroxidase 106
 glycation 122
 glycosaminoglycans (GAGs) 23, 24, 25, 39
 goblet cells 90, 91
 GPCR *see* G protein-coupled receptors
 gradients
 in mesoderm induction 66–71
 mRNA and protein 64–5
growth cone 86, 87
 growth factors 3
 signalling pathways 124, 131, 135, 173
 self-sufficiency 142–57
 receptors 150–1
 growth-inhibitory signals, insensitivity to 157–62, 173
 guanine nucleotide exchange factors (GEFs) 42, 45
 gut
 ageing 107
 development 55, 57, 66
 tumorigenesis *see* colorectal cancer

H

haptotaxis 11, 12
Hayflick limit 109
 heat shock proteins 106, 122, 124, 125
 hedgehog protein family 58
 see also sonic hedgehog
 hemidesmosomes 14, 17
 HER2 receptor 149, 150, 151, 154
 Herceptin 151
 heregulin receptor *see* HER2 receptor

heterologous interactions 9, 15
 heterophilic interactions 15, 22
 HNPCC 136, 174
homeoboxes 77
 homeodomains 77, 81
homologous interactions 9, 15, 16, 22
 homophilic interactions 15, 16, 22
Hox genes 77–80
³H-thymidine DNA labelling 92, 109
 humans
 ageing studies 100, 109–11, 113, 116, 118
 insulin signalling pathway 124
 stem cells 91
 see also cancer
 human adult T cell leukaemia virus (HTLV-1) 145
 human cancer *see* cancer
 human immunodeficiency virus (HIV) 167
 Hutchinson-Gilford progeria syndrome (HGPS) 123
hydrogen peroxide 102–3, 106
hydroxyl radical 103
hyperplasia 134
 hypoxia 2–3, 139

I

I/A domain 18–21, 22
 ICAMs *see* intercellular adhesion molecules
 IGF-1 163, 173
 signalling 112, 124–5
 immortalization 115, 146, 147
immunoglobulin (Ig) supergene family 14, 22–3, 26
in vitro cell culture *see* cultured cells
in vivo studies, senescence 110–11
 inbred strains 100
induction of mesoderm 66–71
 infection, cancer risk 128
 inflammation
 chemokine role 38
 chronic 128
 leukocyte role 9–10, 22, 28, 45
 senescent cells 113
 inherited mutations 136, 137
 cancer risk factor 128, 130, 159, 174–5
 inner cell mass 94
inside-out signalling 20
 insulin signalling pathway 124–5

integrins 14, 17–19

 activation 20–1
 adhesion 22–5, 35, 44
 clustering 29–30
 I/A domain 18–21, 22
 in tumours 171, 173
 intercellular adhesion molecules (ICAM) 18, 19, 22–3
 anchoring proteins 34, 35
 surface expression 29, 30
 amino acid sequence 28
 see also neural cell adhesion molecules; vascular cell
 adhesion molecules
 intercellular communication 5
 intercellular junctions *see* non-junctional adhesion
 interleukins
 and differentiation 3, 94
 interleukin-8 (IL-8) 38, 39, 40
 intestinal epithelial cells 90–3
 ionizing radiation *see* radiation

J

junctional adhesion 13, 14

K

K-ras mutations *see* *ras* gene
 karyotype 132, 136, 137
 Kreisler transcription factor 80
 Krox transcription factor 80

L

L-selectin 25
 labelling 59, 84
lamellipodia 10, 11, 34, 43
 axon guidance 86, 87
 lamins 123
laminin 24–5
 large T protein 161
lectins 25
LEF/TCF 58, 68, 69, 74, 83, 173
leukaemias 134, 141, 143
 chronic myelogenous 153, 154, 155
 leukocytes
 adhesion 13, 28–9, 30
 and cytokines 3
 differentiation 3
 role in inflammation 9–10, 22, 28, 45
 integrin expression 17, 18
 migration 9–10, 25–6, 46

leukotriene B4 (LTB4) 38
 Lewis X ligand (SleX) 10, 26
 lifespan

- different organisms 99
- effect of genes 124–5
- replicative 108–11, 116, 118
- see also* ageing

ligands

- CXC 38–9
- extracellular 14, 17
- G-quadruplex DNA binding 167
- integrins 18, 22
- sialylated Lewis X 26

lipid peroxidation 104, 106

lipofuscin 122

loss-of-function mutations 157–9

lung cancer 129, 154–5, 163

lymphangiogenesis 168, 172

lymphoid tumours 138, 169

M

Mac-1 integrins 18, 19

macrophage 17, 169

- differentiation 3, 94

magnesium ions, integrin–ligand binding 18

malignancy *see* cancer; tumorigenesis; tumour cells

malondialdehyde 104, 107

mammals, cloning 95, 96

mammalian cells

- chemotaxis inhibition 45
- intestinal epithelial cells 90–3
- migration of leukocytes 9–10
- post-mitotic cells 119
- signalling polarization 40
- see also* mouse

mast cells 38

maternal effect genes 65

maternal mRNA 54

matrix metalloproteases (MMPs) 46–7, 112, 171

mdr1 gene 141

melanocytes 82, 85, 134

melanoma cells 134

mesoderm 8, 54, 55, 56

- Hox genes 79, 80
- induction 66–71

metal ion dependent adhesion site (MIDAS) 19–20

metalloprotease disintegrins (ADAMs) 46

metamorphosis 52, 56

metastasis 47, 133, 134, 135, 170–2, 176

microarrays 148

microevolutionary process 131–2, 166

mimetics, SOD/catalase 108

Miranda protein 63

mitochondria

- damage to 119–20, 121
- free radicals 102–3

mitochondrial DNA (mtDNA) 119–20, 121

MLH/MSH genes 136–7

moesin 34

mom gene 64

monoclonal antibodies 151

monocytes 3, 17

morphogen gradients 65

morphogenesis 65

motor neurons, specification 58, 81–2

mouse

- ageing 99, 111, 116, 118
- Hox genes 77, 78, 79
- insulin signalling pathway 124
- intestinal epithelial cells 90–2
- tumorigenesis assays 146, 147

MS cell 61, 62, 64

mtDNA 119–20, 121

multidrug resistance 141

multipotent cells 50, 93

murine mammary tumour virus (MMTV) 146

mutagens 128, 129

mutations

- APC* 174, 176
- from DNA damage 127–32
- gain-of-function 142–8
- genetic analysis 60
- loss-of-function 157–9
- mtDNA 120
- silent 130, 131

mutL and *mutS* bacterial genes 136

myc gene/Myc protein 139, 143, 149, 156, 157, 164, 173

myeloma, multiple, cells 134

myosin II 11, 32, 36

N

- nematode worm *see* *Caenorhabditis elegans*
- nervous system
 - differentiation 71–89
 - see also* vertebrate nervous system
- netrin 87, 88
- 'network theory' 100**
- neural cell adhesion molecule (N-CAM) 22, 84
- neural crest cells 72**
 - migration 9, 58, 82–5
- neural induction 72–4**
- neural plate 52, 56, 74–5**
- neural precursors/progenitors 74, 81**
- neural stem cells, differentiation 93–4
- neurectoderm 56, 72, 73**
- neurite outgrowth 86–8
- NeuroD 74, 75
- neurofilaments 76
- neurogenin 74–5
- neurons 63
 - differentiation 74–5
 - misfolded proteins in 122
 - specification 81–2
- neurospheres 93**
- neurulation 52, 56, 57, 72, 83**
- neutrophils
 - actin polymerization 32, 34
 - differentiation 3
 - see also* leukocytes
- newt limb, regeneration 95
- nitrogen mustard 140, 141
- 3-nitrotyrosine 105**
- Noggin 73**
- non-junctional adhesion 7, 13, 14, 17
- NotI restriction enzyme 159
- Notch proteins 58, 68, 74–5
- notochord 56, 57, 81, 83, 85**
- nuclear transfer experiments 95, 96
- nucleotide excision repair 137

O

- occluding junctions 7, 14
- oestrogen antagonists 151
- oncogenes 131, 135**
 - functional assays 146, 147
 - overproduction of proteins 143, 144
 - see also* proto-oncogenes; viral oncogenes

- oocyte, fertilization 53
- organizer 70, 73–4**
- organogenesis 52, 56**
- ovarian cancer 137
- oxidative damage 103–5, 106, 118
- oxidative metabolism 101–3
- oxidative stress 105, 118**

P

- p53 117
 - in senescence 114–15
 - loss of 164–5, 174, 176, 177
 - tumour suppressor 138–9, 160, 161, 173
- P cells 61, 62, 64
- P-selectin 25, 28
- Paneth cells 90, 91
- papillomaviruses 160
- PAR proteins 62, 63
- paraquat 107, 108
- patterning 56, 58, 67
- p21CIP protein 139, 173
- PDK1 41
- pemphigus 16, 17
- peroxyl radicals 101, 102, 104
- PH domains *see* pleckstrin homology domains
- PhdA (PH domain-containing protein A) 41, 42, 45
- phosphatidylinositols 41, 42, 124
- PI 3-kinases
 - activation 21
 - in IGF-1 pathway 124
 - signalling polarization 40–2
- PKB/Akt 41, 42, 45, 124, 163, 173
- PKC-3 62, 63
- plasticity 89–96**
- platelet-derived growth factor (PDGF) 149–50, 173
- pleckstrin homology (PH) domains 41, 42**
- polyp 134, 174, 175**
- pop* gene 64
- population doubling (PD) 109, 110, 111**
- porphyrins 167
- post-mitotic cells 99
 - ageing of 119–22
 - see also* neurons
- potential clonogenic stem cells 92**
- premature ageing 100, 123
- prenylation 43
- precursor cells 74

presumptive germ layers 54, 55, 66, 67
see also ectoderm; endoderm; mesoderm

primary tumour 170, 171

process, neuronal 73

profilin 33

progenitor cells 74

progerias *see* progeroid syndromes

progeroid syndromes 100, 111, 123

prognosis marker genes 148

proliferative reserve capacity 111

proneural genes 74, 75

prostate cancer 163

proteins

- expression changes in ageing 121–2
- effect of signalling molecules 5–6
- extracellular matrix 23–5
- gradients in development 65
- overproduction in tumours 143, 144, 145

proteoglycans 14, 23, 24–5

proto-oncogenes 131, 135, 143–5

- in growth factor pathways 149–57

pseudopodia 10–11, 32–4

PTEN tumour suppressor gene/*PTEN* protein 163, 172, 173

Q

quadruplex DNA 167

R

Rac 42–3, 44

radiation

- cancer risk factor 128, 137, 138
- intestinal stem cells 92, 111

radiotherapy 140

radixin 34

ras gene 131, 144, 146, 147, 152–3, 174–5

- K-ras* mutations 174, 175, 176

Rb (retinoblastoma) gene

- in senescence 114
- in tumorigenesis 131, 158, 159–61

reactive nitrogen species (RNS) 105, 106, 128

reactive oxygen species (ROS) 101–3, 105, 106, 107, 128

relative risk, of disease 129

replicative senescence 108–19

- overcoming limits 164–7

reprogramming 95–6

restriction landmark genomic screening (RLGS) 159

retinoblastoma gene *see Rb* gene

retinoic acid 79–80, 94

retroviruses 153, 161

- viral assays 146

- viral oncogenes 143–5, 149, 150

reverse transcriptase inhibitors 167

RGD tripeptide motif 23, 24

RGE tripeptide motif 23

Rho-family GTPases 42–3, 44, 58, 84

RhoA 42–3, 44

rhodamine fluorescence 107

rhombomeres 76–7, 80

Robo receptors 45, 88

ROS *see* reactive oxygen species

Rous sarcoma virus 153

RTK (receptor tyrosine kinase) 173

S

SA- β gal reaction 109, 110

sarcomas 134

Scribble protein 63

sea-slug (*Aplysia*), growth cone 87

sea-urchin oocytes 53

secondary tumours 170, 172

segmental progeroid syndromes 123

- see also* progeroid syndromes

selectins 10, 14, 25–6, 29

semaphorin 86, 87, 88

senescent cells

- gene expression 112–13
- mechanism of 113–18
- replicative lifespan 108–11
- see also* adult cells

serine proteases 171

serpins 171

sialoadhesins 26

sialylated Lewis X ligand (sLeX) 26

signalling molecules 5–6, 21, 44, 65

- chemokines 38–40

- cytokines 3, 4

- polarization 40–2

- transcription factors 58

silent mutation 130, 131

skin cancer 137

slime mould *see Dictyostelium discoideum*

Slit family of proteins 45, 88

Slug gene 83–4
Smad4 gene 58, 175, 176, 177
 Smad proteins 58
 smoking, and cancer 128, 129
Snail gene 83–4
somites 56, 83, 85
sonic hedgehog (Shh) 58, 81
Sox genes 74, 75
specification map 60
specified cell 50
 spinal cord 81, 82, 86
 development 76, 78
 sponges, integrins in 17
src gene/Src protein 153, 173
stem cells 50, 90
 adult 93–4, 111
 embryonic 94
 intestinal 91–2, 120, 121
 stem cell factor (SCF) 3
 steroid hormones 95
 stop codon, premature 130
superoxide dismutase 102, 103, 106, 124
superoxide radicals 102, 103, 106
 SV40 virus 160
 sympathetic ganglia 9, 85
syndecans 25

T

tadpole 52
 tailbud stage embryo 52, 96
 talin 21, 35
 tamoxifen 151
tax gene 145
 taxanes 141
telomerase 114, 165–6, 167
 telomeres
 oxidative stress 118
 shortening 113–16, 164–5
 structure 116–17
telomere-dependent pathways of replicative senescence 113
telomere-independent pathways of replicative senescence 113
 TGF β superfamily of proteins 58, 66, 67–8
 in tumorigenesis 160, 173, 177
 thrombospondin 168, 170
thymosin 33
 tight junctions 7, 14

tissue inhibitors of metalloproteases (TIMPs) 46, 171
 α -tocopherol *see* vitamin E
 topoisomerases 141
totipotent cells 93, 94
traction 11
trans-differentiation 95
 transcription factors 80
 dysregulation 156
 neuron-specific 74–5
 signalling pathways 58
 transformed cells 146, 150
 transforming growth factor (TGF) *see* TGF β superfamily of proteins
transit cells 90
 transplantation 60
 TRF2 protein 117
tumorigenesis 127–35
 angiogenesis 168–70
 apoptosis evasion 162–4
 genetic instability 136–42
 growth inhibitory signals 157–62, 173
 growth signals 142–57
 limitless replication potential 164–7
 tissue invasion and metastasis 170–2
 progression to cancer 173–7
 tumours
 evolution 132, 133, 134
 survival of 166
 see also cancer
 tumour cells
 gain-of-function mutations 142–8
 loss-of-function mutations 157–9
 mutations of *ras* 152
 tumour necrosis factor (TNF) 28, 29
tumour suppressor genes 131, 157–61, 163, 176
 Type II diabetes 122
 tyrosine kinases, 149, 150, 154

U

uropod 11, 12

V

v-sis gene 149, 150
 vascular cell adhesion molecule 22
vascular endothelial growth factor (VEGF) 168, 169
 vegetal pole 52, 55, 66, 70
VegT 68, 70

vertebrates

- adhesion molecules 14–15, 16, 17
- chemotactic molecules 38
- neurulation 72
- see also* mammals

vertebrate nervous system

- neural crest cells 82–5
- regional specification 76–80
- spinal cord 81

very late antigen (VLA) 18

villi 90

vinca alkaloids 141

vinculin 35, 43

viral oncogenes 143, 144–5

- in growth factor pathways 149, 150

viruses

- cancer risk factor 128
- tumorigenesis 144–5
- see also* retroviruses

vitamin A 79

vitamin C 106

vitamin E 106

W

Weibel Palade body 28, 39

Werner's syndrome 100, 111, 123

Wnt family of proteins 58, 64, 68–9, 70, 73

- intestinal stem cells 92–3
- in tumour progression 172, 173

Wnt–Frizzled pathway 68, 69, 173

wounds 10, 28

X

xenografts 60

Xenopus laevis

- cloning 96, 97
- development 51, 52, 54, 55, 57
- mesoderm induction 66–71
- neural axis 80
- neuronal development 74–5
- reprogramming 95

Xeroderma pigmentosum 137

Xnr (*Xenopus nodal-related*) genes 68, 69, 70*XP* family of genes 137

Z

Zic genes 74, 75

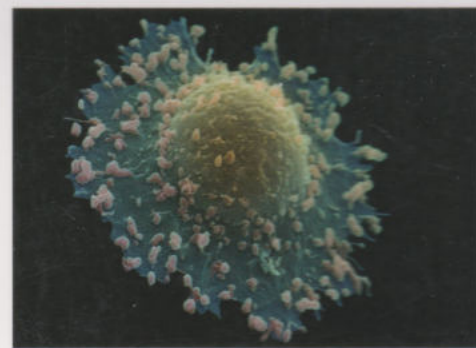
zinc finger proteins 84

zygote 50

zygotic genes 65, 68

- see also* maternal effect genes

S377 Molecular and cell biology
Science: Level 3



Book 1 From Molecules to Cells

Book 2 The Dynamic Cell (Vol. 1)

Book 3 The Dynamic Cell (Vol. 2)

Book 4 The Interactive Cell

ISBN 978-0-7492-2676-3



9 780749 226763